

A fluorescence methodology for assessing the polarity and composition of novel thermoresponsive hydrophilic/hydrophobic copolymer system.

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ABSTRACT

The use of designed polymer coatings for specific applications such as drug delivery or modifying cell response is a critical aspect of medical device manufacturing. The chemical composition and physical characteristics of thin polymer coatings need to be analysed *in-situ* and this can present difficulties for traditional analytical methods. For example, changes in the polarity of polymer coatings are typically measured using the contact angle (CA) method. This is a simple process and gives good results however; it cannot be used to measure very hydrophilic polymers, or to analyse features smaller than a couple of mm in size. There is a need for a non-contact method for polarity measurement that is suitable for hydrophilic polymers on a macro- and microscopic scale.

4'-diethylamino-3-hydroxyflavone (FE), 5, 6-benzo-4'-diethylamino-3-hydroxyflavone (BFE), and 4'-diethylamino-3-hydroxy-7-methoxyflavone (MFE) are fluorescence probes based on 3-hydroxyflavone. They respond to environment perturbations by shift and changes in the relative intensity of two well-separated bands in the emission spectra. These bands originate from an excited state intramolecular proton transfer (ESIPT) reaction. We have incorporated FE, BFE, and MFE into a novel thermoresponsive hydrophilic/hydrophobic copolymer system (NIPAM-NtBA) and studied its fluorescence behaviour. The fluorescence emission spectra depend strongly on copolymer composition, with increasing hydrophobicity (greater NtBA fraction) leading to a decrease in the value of $\log(I_N^*/I_T^*)$. This allows for the non-contact, measurement of the exact composition and surface energy of the copolymer system.

Keywords: Fluorescence; Polymers; Polarity; Surface Energy; drug-eluting polymers, 3-hydroxyflavone.

1.0 INTRODUCTION

Micron scale thin polymer films are a key technology area in modern medical device manufacturing. Significant interest is being invested in designing medical devices coated with drug-eluting polymers, with a view to achieve local delivery of potential anti-restenosis therapy.¹ Polymer stent coatings can serve as drug reservoirs, and as agents to device compatibility within the body.² These polymer coatings are relatively thin (20-100 μm) and formed on small and complex geometries (the typical size of a coronary stent is 15 x 3 mm) and this combination generates significant problems for *in-situ* polymer film analysis. The drug elution rate, long-term storage, device efficacy, and hence regulatory issues are all dependant on the physiochemical properties of the polymer film. Failures in the polymer film

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(irregular crystallite/amorphous domains, lack of adhesion to underlying substrate, irregular drug deposition) can be attributed in many cases to changes in polymer polarity and composition (due to impurities or degree of water uptake).

The analysis of these polymer films for biomedical applications typically involves the analysis of two separate domains, the polymer surface, and the bulk film. Several techniques are currently used to investigating surface properties of thin polymer films,^{3,4} the most popular of which is the contact angle (CA) method, which is used to give a measure of surface energy.⁵ CA is a simple method and gives good results, however, it cannot be used to measure very hydrophilic polymers when water is used as a solvent. However, water CA is a very important characterisation parameter for surfaces of biomedical devices.⁶ If organic solvents are used instead of water then other problems can be encountered such as polymer dissolution, solvent infiltration, chemical reaction between solvent and polymer, and a lack of solvents with the appropriate surface tension. Another limitation of CA is the difficulty in obtaining measurements on complex features smaller than a couple of mm in size.

For analysis of the bulk properties of polymer films *in-situ*, various optical techniques can be employed including vibrational, fluorescence, and UV-Visible absorption spectroscopy of solvatochromic probes (UVASSP).^{7,8} There are several issues with the use of UVASSP for polymer analysis, which include the low inherent sensitivity of absorption spectroscopy, the need for specialist instrumentation, and the use of single-band solvatochromic dyes. Using classical single-band solvatochromic dyes, one cannot obtain sufficient spectroscopic data for calculating all the various physicochemical parameters simultaneously. In most cases the analysis of probe response is provided on a monoparametric scale, that is, finding a correlation between a single spectroscopic parameter (usually the shift in the absorption or fluorescence spectrum) with some empirical scale (e.g. Et(30) for solvent polarity⁹). Fluorescence methods are inherently more sensitive than absorption methods, and there is wide scale use of fluorescent probes for monitoring the local environment of materials and biological systems.¹⁰ However, for a fluorescence method to be useful for characterising polymer films it must be able to overcome problems such as photobleaching, excitation source instabilities, and local variations in probe concentration. One way in which this can be achieved is by using ratiometric fluorophores, which are in essence, self-calibrating and immune to the difficulties associated with the use of single band fluorescence measurements. One such class of ratiometric fluorophores that have recently been developed are based on 3-hydroxyflavone (3-HF).^{11,12}

These 3-HF probes operate on the principle of excited-state intramolecular proton transfer (ESIPT) and exhibit very strong solvatochromism and electrochromism. In contrast to many other fluorescence probes, they sense the polarity or electric fields by spectral shifts, and also by the redistribution of intensity between two well-separated emission bands.¹³ One of these bands belongs to the normal excited state (N^*), and the other to the ESIPT reaction product tautomer (T^*). An important parameter is the ratio of intensities of the N^* and T^* bands in emission, I_N/I_T , which is connected to the relative energies of the N^* and T^* states¹⁴ and is a very sensitive indicator of solvent polarity.¹⁵ The sequence of events that results in two emission bands for the FE probe is depicted on Figure 1.

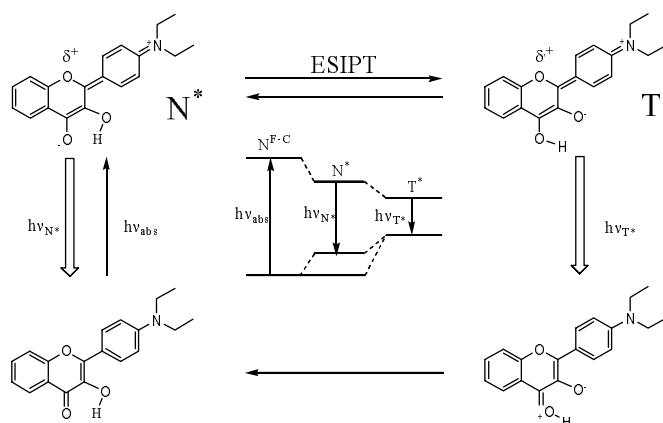


Figure 1: ESIPT photophysical cycle for the flavone fluorophore 4'-diethylamino-3-hydroxyflavone (FE).¹³

A number of spectroscopic variables can be recorded each of which is sensitive to different kinds of perturbation of the local environment.^{13,16,17,18} The spectroscopic behaviour of these bands as well as the interplay of their intensities depends strongly on the structure of the probes. Changing the chemical structure at the core of fluorophore can be used to: adjust the dye to specific range of solvent polarity, switch off the sensitivity to hydrogen bonding, or introduce ion-chelating groups.^{19, 20}

There is a need for a non-contact, non-destructive, and sensitive method for polymer polarity measurement that is suitable for hydrophilic polymers on both macro- and microscopic scales. Therefore we have started developing a fluorescence-based methodology for the quantitative analysis of polymer coatings (in particular drug-eluting types) using a range of 3-hydroxyflavone (3-HF) based fluorescent probes. In this work we have incorporated these fluorophores into a novel thermoresponsive hydrophilic/hydrophobic copolymer system, NIPAM-NtBA (Figure 2). These copolymers are being developed as potential drug elution matrices and for cell culture applications.^{21, 22} The goal of this work is to provide a non-contact, non-destructive fluorescence method for measuring the composition and surface energy of these relatively hydrophilic polymers.

2.0 MATERIALS AND METHODS

A series of copolymers of N-isopropylacrylamide (NIPAM) and N-tert-butylacrylamide (NtBA) with ratios of 100:0, 85:15, 65:35, 50:50, and 0:100 were provided by Dr. Y.A. Rochev and Dr. A.V. Gorelov [Figure 2]. These polymers were used without any further purification.

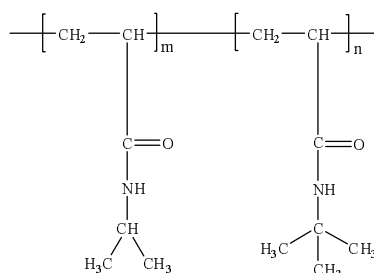


Figure 2: Chemical structure of PNIPAM/PNtBA copolymers showing the very small difference of one methyl group.²²

The fluorescence probes 4'-diethylamino-3-hydroxyflavone (FE), 5,6-benzo-4'-diethylamino-3-hydroxyflavone (BFE), and 4'-Diethylamino-3-hydroxy-7-methoxyflavone (MFE) [Figure 3], were obtained from Dr. Andrey S. Klymchenko and used as received.^{13,17,19}

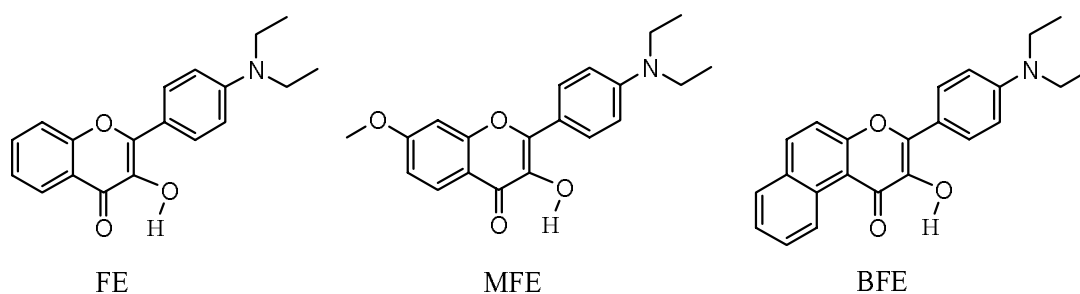


Figure 3: Chemical structures of the fluorescent flavone based fluorescent probes used in this study.

Ethanol of spectroscopic grade was purchased from Sigma-Aldrich and used as received. Quartz slides 12 mm x 45 mm x 1.5 mm (Lightpath Optical Ltd., UK) were used as a solid substrate for copolymers film. The slides were washed carefully at least four times with deionised water, acetone, and methanol, and dried in an oven at 70°C before use.

If a polymer film is to be used as a drug delivery medium then it must be of sufficient thickness to accommodate the requisite amount of the drug. It is generally understood that a minimum thickness of about 5 to 10 μm is necessary to facilitate adequate loading of therapeutic agents into the polymer coating.²³ In our case, to make films of ~ 10 μm (after drying) in thickness, a 137.5 μL aliquot of a 5% solution of the polymer in a 5.7×10^{-5} M ethanol solution of the requisite probe was spread on a quartz slide (12 mm x 45 mm). The films were cured for 24 hours in a sealed environment with a source of ethanol, in order to eliminate any traces of water in the air. After 24 hours, the copolymer films were removed from the ethanol environment and placed into an oven at a temperature of 70°C for 48 hours to complete the drying process. The copolymer films thus obtained were transparent, smooth, and free of any physical inhomogeneities and surface corrugations.

All optical measurements were made at room temperature ($\sim 20^\circ\text{C}$) under normal ambient conditions. Steady state fluorescence spectra were obtained using a Cary Eclipse (Varian) spectrophotometer, fitted with a front surface sampling accessory with slits set to 5 nm for emission and 2.5 nm for excitation spectra measurements. Fluorescence lifetimes were recorded using the Time Correlated Single Photon Counting (TCSPC) method with fluorescence lifetime spectrometer (FluoTime 200, PicoQuant, Germany). Fluorescence was excited using Light Emitting Diode with centre wavelength of 380 nm, which was pulsed using a PDL-800B laser/LED driver (PicoQuant, Germany) at 20 MHz. The pulsed excitation light was filtered to remove any spurious long wavelength emissions. Measurements were made at a count rate of less than 1% of the pulse rate to maintain Poisson statistics. The instrument response function (IRF) was collected under the same conditions as the sample using a clean quartz slide. Fluorescence lifetimes were extracted from the measured decay curves using the FluoFit program (PicoQuant, Germany), which implements nonlinear least-squares error minimization analysis, based on Simplex and the Levenberg-Marquardt algorithms. The final quoted result was determined by the fit, which had a χ^2_R value of less than 1.2 and a residual trace that was symmetric about the zero axes.

3.0 RESULTS & DISCUSSION

In the present study we started from the known fact that fluorescence emission of the FE, MFE, and BFE probes are extremely sensitive to the properties of solvent environment.^{13,17,19} An increase in solvent polarity and hydrogen bonding ability of the solvent environment leads to an increase of the N^* form relative to T^* form, which is due to a greater dielectric stabilization of N^* form.¹³ By measuring the ratio of the intensities of these two forms, a method can be developed to measure the changes in polarity and hydrogen bonding. This has already been done for solvents,^{13,19} and so by extension, placing these fluorophores in polymers and measuring the intensity ratio between N^* and T^* bands should provide an analogous method for measuring polymer polarity. In the case of the NIPAM/NtBA copolymer system, the change in polymer polarity should be directly correlated with the copolymer composition. This would be useful in analysing these films *in-situ* on medical devices. In solvents these measurements are relatively straightforward since the fluorophore's local environment will be homogeneous. The polymer case however, is more complex since unlike ideal crystals, polymers display structural, energetic, and dynamic microscopic inhomogeneity. In the case of NIPAM/NtBA thermoresponsive polymers, which are random linear copolymers, there is the added possibility of local hydrophilic/hydrophobic microenvironments.²²

The fluorescence emission spectra from all 3-HF fluorophores are strongly dependant on copolymer composition, with increasing hydrophobicity (greater NtBA fraction) leading to a decrease in the ratio between the emission intensity from the N^* and T^* bands ($I_{\text{N}^*}/I_{\text{T}^*}$) as shown in Figure 4A, 4C, 4E. It is interesting to note in these spectra that the positions of the N^* and T^* bands do not vary with copolymer composition, despite the large separation between the bands. The corresponding excitation spectra [Figure 4B, 4D, 4F] show a blue shift of the T^* band relative to that of the N^* band, but that there is no difference in excitation wavelength maxima between the PNIPAM and PNtBA polymers. In a more homogeneous solvent case (ethanol) the excitation spectra are identical for the N^* and T^* bands (data not shown).²⁷

Indeed for the N* band the excitation spectra are identical in both cases (for PNIPAM and PNtBA) indicating that polymer composition has no influence on the N* state. In the T* case the overlap is not quite as perfect, and while the maxima are the same for PNIPAM and PNtBA, the red edge of the excitation spectra is red-shifted for PNIPAM relative to PNtBA. Comparing the excitation spectra for all 3 probes shows that there is no significant difference in the separation of the two excitation spectra (all ~10 nm) indicating that the magnitude of the separation effect is largely independent of the nature of the probe. This would suggest that if it is a hydrogen bonding interaction that causes these changes, it must occur via the carbonyl of the polymer and the 3-hydroxy group of the probes.²⁴

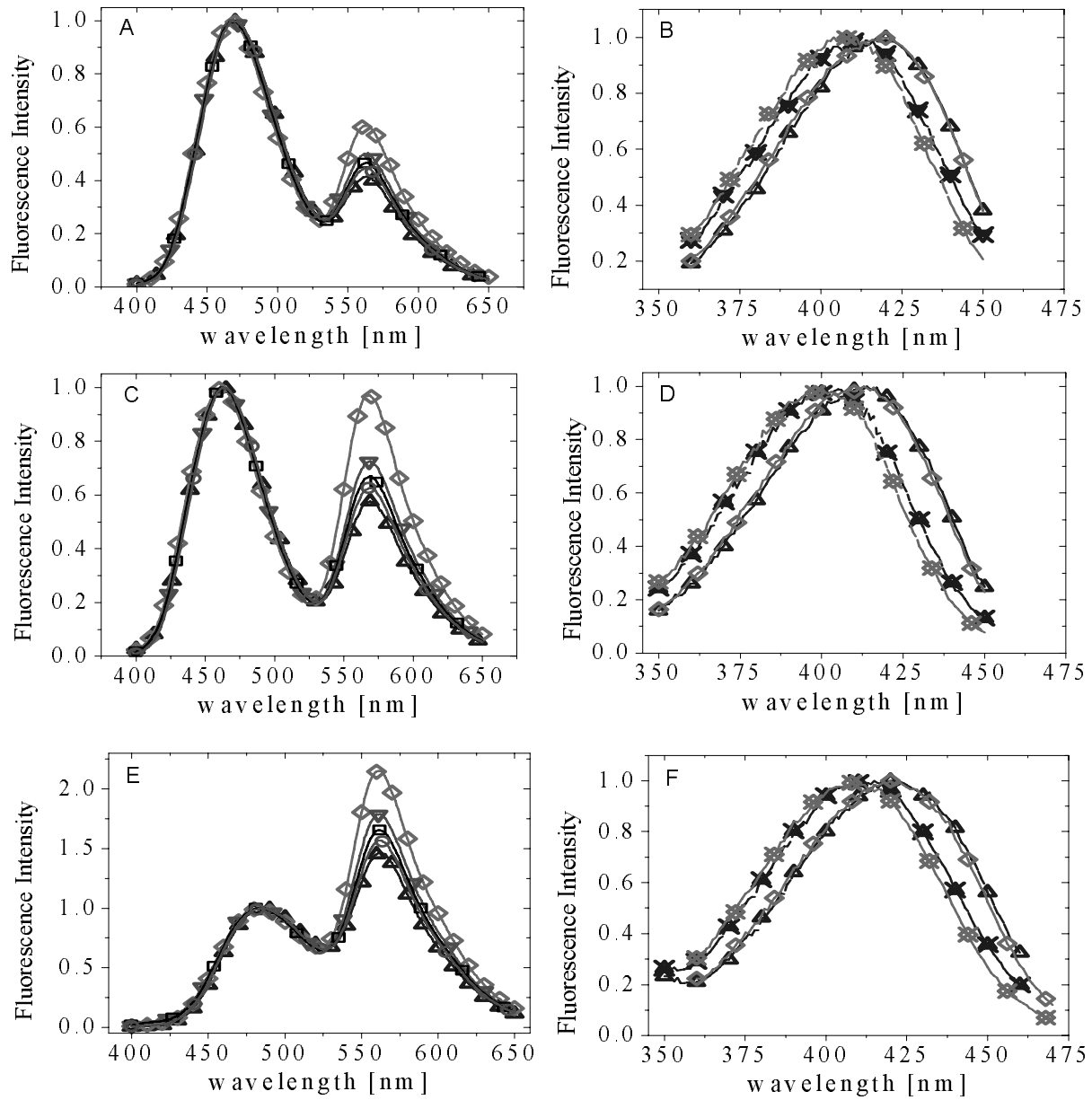


Figure 4: Normalized fluorescence spectra (A, C, E) and excitation spectra (B, D, F) of hydroxyflavone FE (A & B), MFE (C & D) and BFE (E & F) doped copolymers. (A, C, E): PNIPAM (Δ), P85 (\circ), P65 (\square), P50 (∇), PNtBA (\diamond), excitation wavelength 375 nm. (B, D, F): excitation spectra of PNIPAM (Δ), (\otimes) and PNtBA (\diamond), (\otimes) recorded at maximum of N* (Δ), (\diamond) and T* (\otimes), (\otimes) band.

It has been observed that for a related 3-HF fluorophore (4'-(dimethylamino)-3-hydroxyflavone) in solvent mixtures containing a proton donor that the excitation spectra of the N* form is also red-shifted relative to the T*.²⁵ This is important in the context of developing a quantitative measure for the polarity of the polymer matrix.

Comparing the change in band intensity ratios (using the $\log(I_{N^*}/I_{T^*})$ value) versus the composition of the copolymer (mol % NtBA) [Figure 5A] shows a good linear correlation for each of the 3 probes with the PNIPAM being more polar than the PNtBA. The NIPAM and NtBA constituents of these copolymers differ only by one methyl group [Figure 2] and this small difference is very difficult to observe using other spectroscopic methods, and in the case of FT-IR is impossible.²⁶ Each of the dyes can thus be used for polymer composition measurements but MFE shows the greatest change in the $\log(I_{N^*}/I_{T^*})$ parameter making it the most sensitive. In aprotic solvents, the FE and BFE probes show very similar spectroscopic properties, since there is no hydrogen bonding contribution from the solvent. In protic solvents they show different behavior due to intermolecular hydrogen bonding with the 4-carbonyl group. Therefore, the ordering of the plots in Figure 5A from top to bottom, is a result of decreasing H-bonding contribution, with BFE having a much reduced H-bonding ability due to the influence of the additional aromatic ring in its structure Figure 3. Table 1 gives the results of the linear fits to the data.

The next step in developing the methodology was to correlate surface energy data for these copolymers with the fluorescence data. It has been discovered that there is a non-linear correlation between polymer composition and surface energy as measured by tedious contact angle measurements.⁶ If we plot surface energy vs. $\log(I_{N^*}/I_{T^*})$ for the BFE loaded copolymers (to minimise H-bonding effects) we get a very clear exponential dependence [Figure 5B] which reaffirms that PNIPAM is more polar than the NtBA containing copolymers.

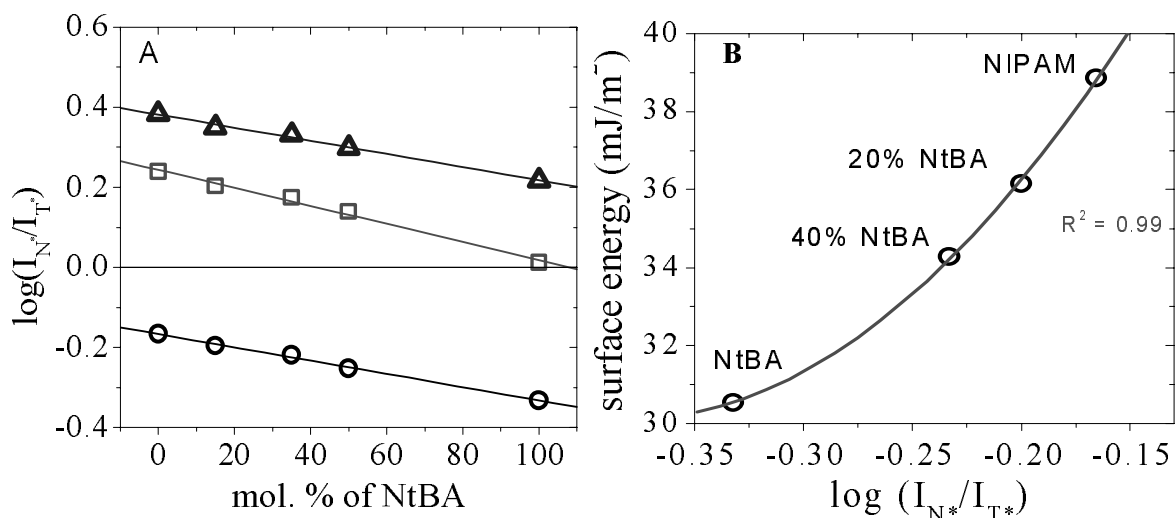


Figure 5: (A) Dependence of $\log(I_{N^*}/I_{T^*})$ on mol. % of hydrophilic component NtBA for the hydroxyflavone (FE (Δ), BFE (\circ), MFE (\square)) doped films, excitation wavelength 375 nm. (B) Surface energy (⁶) vs. $\log(I_{N^*}/I_{T^*})$ of hydroxyflavone BFE doped copolymers. Excitation wavelength 375nm. Surface energy data obtained from references 6 and 22.

| Fitting equation : $y = bx + a$ | | | | |
|---------------------------------|------------------------|----------------------------|-------|--------|
| Hydroxyflavone | a | b | SD | R |
| FE | 0.382 ($\pm 0,004$) | -0.00164 ($\pm 8E-5$) | 0.006 | -0.996 |
| MFE | 0.244 ($\pm 0,006$) | -0.00225 ($\pm 1.18E-4$) | 0.009 | -0.996 |
| BFE | -0.167 ($\pm 0,003$) | -0.00165 ($\pm 5,7E-5$) | 0.004 | -0.998 |

Table 1: Fit results for data in figure 5A: Dependence of $\log(I_{N^*}/I_{T^*})$ on mol. % of hydrophilic component NtBA for hydroxyflavone doped copolymers.

The next step in this study was to utilize the probes for quantitative polarity measurements. The proposed model for use with these dyes has been developed by Klymchenko and co-workers for use in solvents.¹³ It correlates solvent polarity function ($f(\epsilon)$) with two parameters, the $\log(I_{N^*}/I_{T^*})$ value and the band separation. Plotting these two parameters yields a linear plot in the solvent model from which $f(\epsilon)$ can be then be calculated.

However, when we investigated the effect of different excitation wavelengths on the 3-HF probe loaded copolymers we found that the intensity ratio (I_{N^*}/I_{T^*}), Figure 6A-C, and the position of N^* band varies for every dye, Figure 7. In solvents, this excitation dependence is not observed.²⁷ The explanation for this excitation wavelength dependence, must reside in the interaction between the fluorescence probe and the copolymer matrix.

There are two contributing factors, (1) a major hydrogen bonding effects and (2) a minor red edge effect:

- 1). The NIPAM-NtBA copolymer system is relatively polar and can act as both a H-bond donor and a H-bond acceptor. From the fluorescence emission data we can conclude that there is a significant H-bonding interaction between the probes and the copolymers. There are two possibilities, first between the 4-carbonyl group of the 3-HF fluorophore and the amide hydrogen (N-H) of the copolymer [Figure 4B, 4D], and second between the 3-hydroxy group of the probe and the carbonyl of the polymers. The first case is equivalent to the protic solvent case and would be expected to lead to stronger N^* emission compared to T^* , and a smaller band separation. The H-bonding influences ESIPT by slowing down or preventing the process which leads to weaker, T^* emission. It was shown that in protic solvents, the ESIPT of FE and MFE probes is strongly hampered compared to that in aprotic solvents of similar polarity (i.e. higher value of I_{N^*}/I_{T^*} ratio).^{13,17} The second interaction is more akin to the aprotic solvent case where one expects larger band separations and weak N^* emission.¹ However, for FE and MEF we observe no change in band position, a large band separation, and a strong N^* emission. Therefore we can conclude that the effects are due to a combination of both types of hydrogen bonding, and this could explain why we observe such a large separation between N^* and T^* bands, and at the same time relatively strong N^* emission. This would also explain why the polymer results do not behave like solvents where there is linear dependence between $\log(I_{N^*}/I_{T^*})$ and band separation $\nu(N^*) - \nu(T^*)$.¹³ In the case of BFE, it has a much lower threshold for hydrogen bonding via the probe carbonyl bond due to the presence of the aromatic ring in the 5,6 position, but still allows the intramolecular bond with the 3-hydroxyl group, which forms the ESIPT pathway.¹⁹ However, we still have hydrogen bonding via the 3-hydroxy group and the carbonyl group of the polymer, which leads to the excitation wavelength dependence [Figure 6C]. Elimination of the effect connected with H-bonding by 4-carbonyl leads to a decrease in the I_{N^*}/I_{T^*} ratio, but a linear trend is still observed for $\log(I_{N^*}/I_{T^*})$ vs. copolymer composition [Figure 5].
- 2). There is also a small contribution from classical red-edge effect which is can be observed in rigid media.^{28,29} In this case red-edge excitation will photo-select dielectrically stabilized species, which shows lower T^* emission. Thus, red edge excitation results in the decrease in the T^* band relative intensity, yielding a higher value of I_{N^*}/I_{T^*} ratio.

The influence of hydrogen bonding, both donor and acceptor, between the copolymer matrix and the probes makes it impossible to calculate the polarity function ($f(\epsilon)$) for this type of polymer environment because it is not possible to separate out the hydrogen bonding component, even when using BFE. Therefore the intensity ratio parameter

¹ There is very little data available on the effect of the 3-HF probes acting as H-bond donors.

$\log(I_N^*/I_T^*)$ therefore gives us information about the total polarity as the sum of both dipole-dipole and specific hydrogen bonding interactions. This will be useful for the characterization of biomedical polymers.

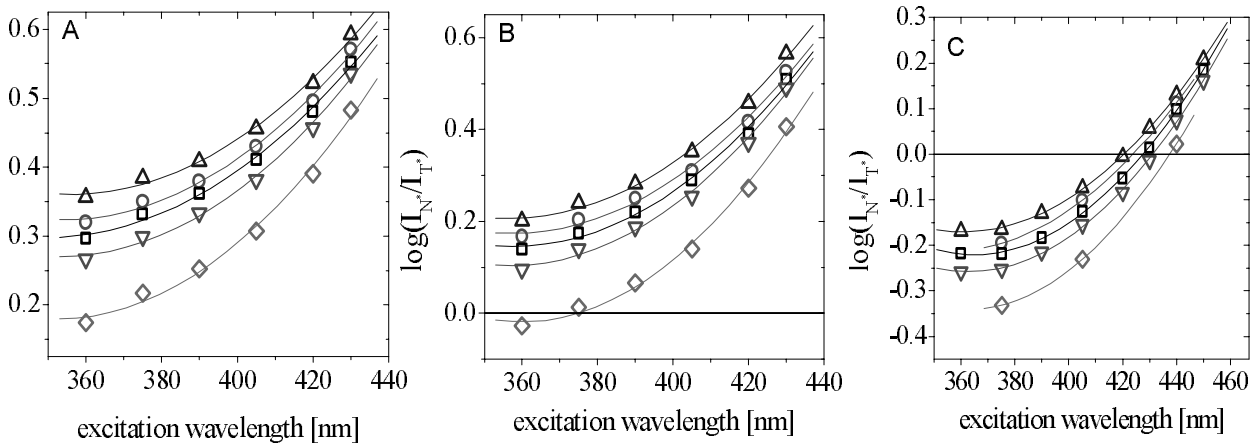


Figure 6: Dependence of the spectral parameter $\log(I_N^*/I_T^*)$ on excitation wavelength. Flavone FE (A), MFE (B), BFE (C). PNIPAM (Δ), P85 (\circ), P65 (\square), P50 (∇), PntBA (\diamond).

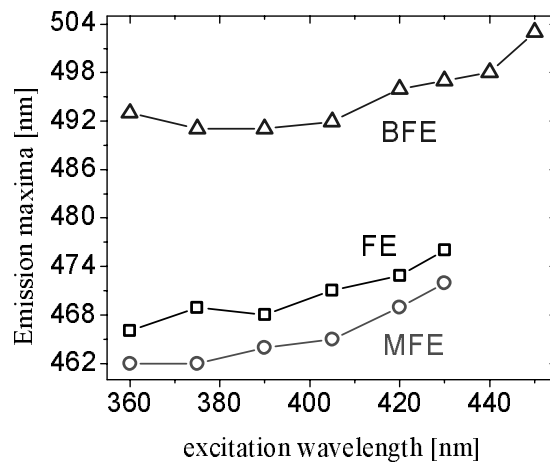


Figure 7: Position of N^* band maxima in NIPAM-NtBA copolymer system (50 % NtBA case) as a function of excitation wavelength.

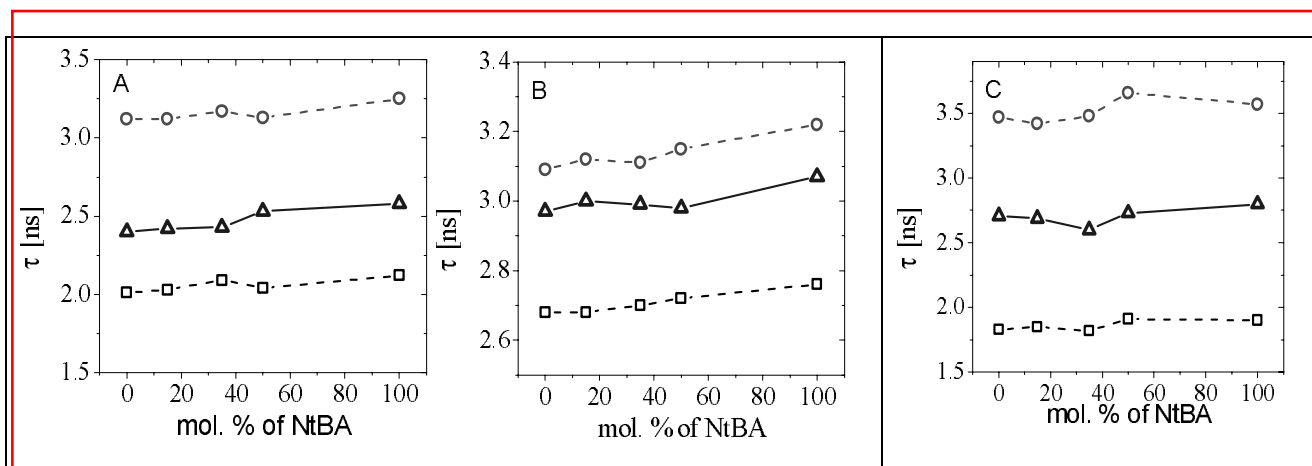


Figure 8: Average fluorescence lifetime ($\bar{\tau}$) of flavone FE (A), BFE (B) and MFE (C) doped copolymers measured at maximum of N* band (\square), maximum of T* band (\circ), all emission collected (long pass filter at 410nm).

We also investigated the fluorescence lifetimes of these dyes in the copolymers. This was done to see if there was a correlation between polymer composition and lifetime, and to provide more fundamental information on the ESIPT process in the heterogeneous environments of the copolymers.

Figure 8 shows the intensity-averaged lifetime ($\bar{\tau}$) data obtained for all 3 dyes in each of the copolymer films. However, the fluorophores do not show any significant changes in the intensity-averaged lifetime ($\bar{\tau}$) with changes in copolymer composition at the emission wavelengths corresponding to the N* or T* bands. There are differences in intensity between the N* and T* bands, but when we collect the complete emission, these differences do not have a very large effect on $\bar{\tau}$ overall. Therefore lifetimes cannot be used for composition measurement.

In aprotic solvents such as ethyl acetate and dichloromethane the lifetimes for the N* and T* bands are equal, which indicates that ESIPT is a fast, reversible two-state process.³⁰ In the copolymer system this is not the case and there is a significant difference in lifetime for the N* and T* bands indicating a non-equilibrated ESIPT system. This is obviously due to a hydrogen bonding interaction between the polymer and the excited N* and T* species. A similar effect was observed in the parent 3-hydroxyflavones in protic solvents where the solvent hydrogen bonding with the dye may produce an activation energy barrier to ESIPT.³¹ In this case ESIPT is irreversible and is characterized by the fact that the lifetime of the T* band is longer than that for the N* band.^{30,31} The most non-equilibrated ESIPT seems to occur in MFE doped copolymers where the difference in lifetime ($\Delta\bar{\tau}$) is ≈ 1.6 ns while the BFE doped copolymers have a much lower $\Delta\bar{\tau}$ of ≈ 0.4 ns [Figure 8A-C]. This supports the argument that the interaction is at least partly due to hydrogen bonding effects since the BFE probe is inherently less suitable for hydrogen bonding at the carbonyl group due to the presence of the additional aromatic ring.

4.0 CONCLUSIONS

Preliminary results show that fluorescent 3-hydroxyflavone derivatives can be used to easily measure changes in composition of random-linear, hydrophilic/hydrophobic thermoresponsive copolymers. This offers a fast and facile method for measuring the copolymer composition. In addition the intensity ratio value of $\log(I_{N^*}/I_{T^*})$ correlates very well with surface energy measurements obtained from tedious contact angle methods. However, we also observe an excitation wavelength dependence, which is due to hydrogen bonding between the probes and the polymer matrix. This interaction prevents us at this stage from obtaining a numerical value for the polarity function of the various copolymers from the fluorescence emission data according to the Klymchenko model.¹³ We are continuing to develop this methodology further with a view to producing a quantitative method for characterising polymers relevant to biomedical applications.

5.0 ACKNOWLEDGEMENTS

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