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FLUORESCENCE ANALYSIS OF THERMORESPONSIVE POLYMERS.

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1 ABSTRACT.

The use of microscale thin polymer films is widespread in biomedical science and engineering, with applications in areas such as tissue engineering, drug delivery, microfluidic devices, bio-adhesion mediators, and bio-actuators. Much attention is devoted to the use of functional polymers that display stimuli-responsive behavior with the intention of providing "smart" coatings. One potential example is the use of thin thermoresponsive polymer films as drug eluting coatings on medical devices, where not only does the polymer function as a drug reservoir but it also acts as a biocompatibility modulator to improve device performance.

Often these thin polymer coatings have to be applied to complex geometries, which can cause problems for in-situ analysis. Another important consideration is the fact that these films have large surface area to mass ratios and thus water uptake can be significant. This is serious because coating stability, device efficacy, and long-term storage are influenced by the physiochemical properties of the polymer which are modulated by water content. Thus, there is a need for a rapid, non-contact, non-destructive, analytical method capable of analyzing thermoresponsive polymers in solution, and in-situ of the solid-state on medical devices. Fluorescence spectroscopy based methods can deal with both sample types and provide additional benefits in terms of high sensitivity and low probe concentrations, which provide for minimal sample disruption. This article gives a brief overview of the application of various fluorescence methods for the physicochemical characterization of thermoresponsive polymers such as poly(N-isopropylacrylamide), PNIPAm.

2 INTRODUCTION.

The use of microscale thin polymer films is widespread in biomedical science and engineering, with applications in tissue engineering, drug delivery systems, microfluidic devices, bio-adhesion mediators and bio-actuators [1-14]. The choice of polymer for such applications is very important, and one area of significant interest has been the development functional polymers that display stimuli-

responsive behavior with the intention of providing "smart" applications (Figure 1) in the biomedical field [8,15,16]. One potential use of thin thermoresponsive polymer films is as coatings on drug eluting coronary stents, where not only does the polymer function as a drug reservoir (providing anti-restenosis therapy) but it also acts as a biocompatibility modulator to improve device performance [5,10,17,18].

In many applications, these polymer coatings are very thin (from μm to nm) and are formed on complex geometries, which may cause problems for *in-situ* analysis. Another important consideration is the fact that these films have large surface area to mass ratios and water uptake is an important factor to consider. This is serious because issues such as device manufacturing, coating stability, device efficacy, and long-term storage are influenced by the physiochemical properties of the polymer [19]. Thus, there is a need for the non-contact, non-destructive, analysis of these types of thermoresponsive polymers in solution and *in-situ* for fabricated films/devices. Optical spectroscopy, and in particular fluorescence based methods, which offer the combination of high sensitivity and low probe concentrations, provide the best solution for these analytical challenges

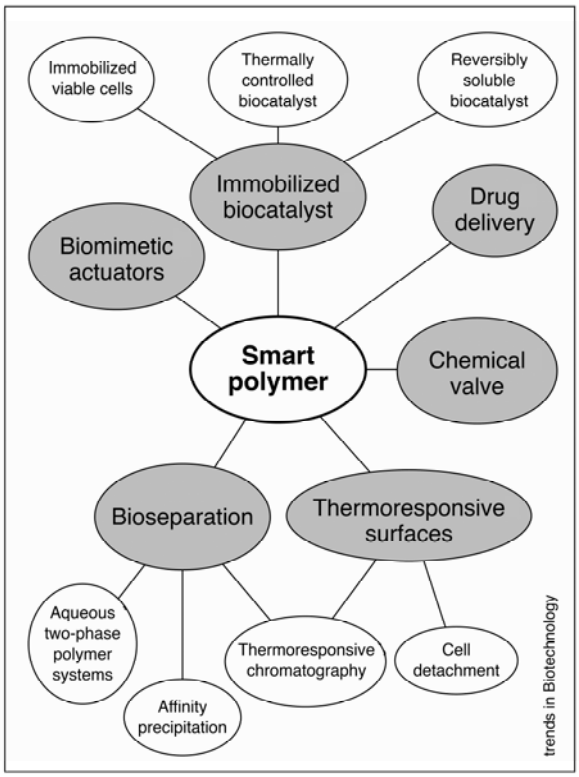

Figure 1: Potential uses of stimuli-responsive polymers in biotechnology and medicine. Adapted with permission from ref. [15]. *Copyright © 1999 Elsevier Science Ltd., all rights reserved.*

This article gives a brief overview of the application of fluorescence methods to the characterization of thermoresponsive polymers and in particular poly(N-isopropylacrylamide), PNIPAm. It is not meant to provide a comprehensive or detailed review of the use of fluorescence

but rather an insight into how various fluorescence methods can be employed for studying the physical and chemical properties of thermoresponsive polymers in a variety of states and forms.

3 STIMULI-RESPONSIVE POLYMERS.

The design, synthesis, and development of functional polymers that respond to external stimuli is an area of significant interest [8,15,16,20]. These synthetic polymer systems are often designed to mimic natural biopolymers, and a variety of functional forms have been developed to meet various specific biomedical and scientific applications [21]. These polymers are variously described as being "environment sensitive polymers" [22], "stimuli-responsive polymers" [23], "intelligent polymers" [24,25], or "smart polymers" [15,26]. "Smart" polymers can be defined as materials that undergo strong conformational changes in response to small changes in the surrounding environment [15,21].

In many of the smart polymers used for biomedical applications, the sharp, large, reversible non-linear conformational changes can be attributed to the balance between hydrophilic and hydrophobic groups within the polymer system [8,15,16,23,27-29]. The consequent responses may be observed as changes in shape, solubility, and/or surface characteristics [21]. "Smart" polymers can be classified into three types depending on their physical forms (Figure 2): *(i)* linear free chains in solution (following the application of an external stimulus the polymer undergoes a reversible collapse), *(ii)* covalently cross-linked gels and reversible or physical gels (for which swelling or shrinking behavior is environmentally triggered), and *(iii)* chain adsorbed or surface-grafted form (following a change of the external parameter, the polymer reversibly swells or collapses on a surface) [21].

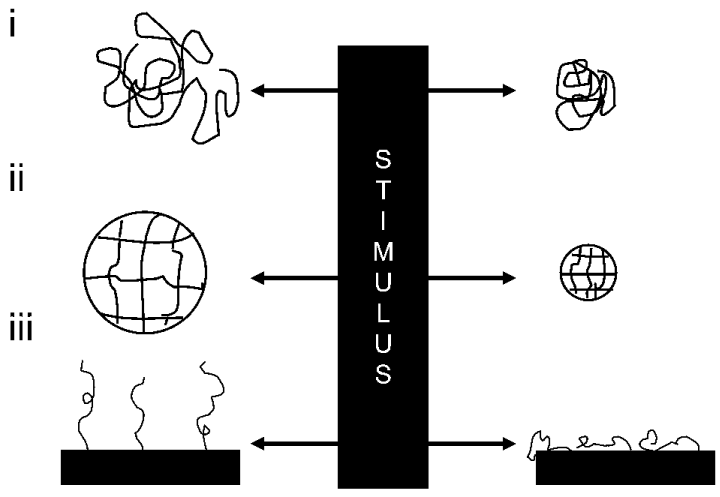

Figure 2: Classification of the polymers by their physical form: (i) linear free chains in solution (ii) covalently cross-linked reversible gels (iii) chain adsorbed or surface grafted form. Reproduced from Kumar *et al.* [21] with permission.

The response of these "smart" polymers can be induced by a variety of environmental triggers, such as ionic strength, light, magnetic field, pH, electric field and temperature [1,8,15,16,20,27,29-37]. From a biomedical standpoint, the favored "smart" polymers are generally those sensitive to pH and/or temperature changes [30]. The most widely studied class of stimuli responsive polymers are thermoresponsive polymer systems; as the name suggests, these systems undergo conformational changes in response to temperature [16,30].

3.1 THERMORESPONSIVE POLYMERS.

Thermoresponsive polymers have a critical solution temperature, at which a significant phase change occurs. Polymers whose water solubility increases with temperature are described as having an upper critical solution temperature (UCST) or a higher critical solution temperature (HCST) and solutions of these polymers appear biphasic below this critical temperature. Conversely, polymers for which the solubility decreases with increasing temperature often have a lower critical solution temperature (LCST) where the solutions transition to a biphasic state. This occurs because the polymer becomes less solvated as the temperature increases [16,22,30,38]. Below the LCST, the polymer is soluble in aqueous solutions due to the domination of hydrophilic interactions (*i.e.* hydrogen bonding between the polymer and water) over hydrophobic (intramolecular) interactions, and thus it typically assumes a relaxed coil-like conformation. Raising the temperature above the LCST results in an increased dominance of the hydrophobic interactions, causing the collapse/contraction of the polymer. This leads to the adoption of a more globule-like conformation which minimizes the polymer-water contact and can eventually lead to precipitation from solution [30,39,40].

In the case of polymer solutions with a UCST, the entropy of mixing is usually large and positive but is dominated by enthalpic contributions at low temperatures. When the temperature increases, the entropic contribution increases, and eventually surpasses the enthalpic contribution at the UCST, resulting in a negative Gibb's free energy. Therefore in these polymer systems, higher temperatures enhance solubility [38]. For polymer solutions possessing a LCST, H-bonding between polymer polar groups and water molecules are the driving force for solvation at low temperatures, resulting in a large, dominant, negative enthalpy of mixing. In this state, the polymer is ordered, leading to an unfavorable negative entropy contribution, but overall the system is stable in this mixed form below its LCST due to the large enthalpic contribution. It has been suggested that the phase separation at the LCST of a polymer can be attributed to entropic effects [41]. At higher temperatures the contribution from entropy (displacement of water from the polymer matrix) surpasses the exothermic enthalpy contribution from hydrogen bonding between polar groups in the polymer and water molecules [8,27,30,41,42]. It is this balance between entropy and enthalpy that causes the polymer

to assume a more hydrophobic state above the LCST.

Abbreviation	***Name***	***LCST (℃)***
PPO	poly(propylene oxide)	10-20
PNPAm	poly(N-n-propylacrylamide)	25
PNIPAm	poly(N-isopropylacrylamide)	32
PEPA	poly(ethoxypropylacrylamide)	~32
PVME	poly(vinyl methyl ether)	33.8
PIPOZ	poly(2-isopropyl-2-oxazoline)	~36
PVCL	poly(N-vinylcaprolactam)	38
HPC	Hydroxypropylcellulose	42
PBMEAm	poly(N,N-bis(2-methoxyethyl) acrylamide)	49
MC	Methylcellulose	50
PDMA	poly((2-dimethylamino)ethyl methacrylate)	50
PEOZ	poly(2-ethyl-2-oxazoline)	~62
PMPAm	poly(N-(3-methoxypropyl)acrylamide)	>60
EHEC	Ethyl(hydroxyethyl)cellulose	65
PEMA	poly(N,N-ethylmethylacrylamide)	70
PEA	poly(N-ethylacrylamide)	82

Table 1: LCSTs of some common thermoresponsive polymers [40].

A list of popular thermoresponsive polymer systems with their corresponding LCSTs is given in Table 1. PNIPAm and PVCL exhibit LCST behavior in a physiologically relevant temperature range rendering them viable for many biomedical applications [21,40,41]. Often these polymers are used as core elements to synthesize co-polymers with specific thermoresponsive and other desired properties. For example, PEO and PPO have been used to fabricate block copolymers, which possess an inverse thermoresponsive behavior. These block copolymers are available commercially under the names *Pluronics*® and *Tetronics*®, many of which have been approved by the FDA and EPA for use in food, pharmaceuticals and agriculture [8,21,22]. Alternatively copolymerization with hydrophilic or hydrophobic co-monomers can be used to increase or decrease the LCST respectively [36]. It is also possible to modulate or fine tune the LCST by the addition of additives for example, the addition of sodium dodecylsulfate increases the LCST, whereas addition of sodium chloride has the opposite effect [43,44].

3.2 POLY(N-ISOPROPYLACRYLAMIDE), PNIPAM.

Of the family of poly(N-substituted acrylamides), PNIPAm (Figure 3) is probably the most widely known and studied. PNIPAm is a chemical isomer of poly-leucine, in that it has the polar peptide group in its side chain rather than in the hydrocarbon backbone [45]. In aqueous solution, PNIPAm has a LCST of 32°C; at this temperature it undergoes a sharp and reversible coil-to-globule phase transition from a hydrophilic to a more hydrophobic state, forcing water from the matrix [3,7,14,16,27,46-48]. This phenomenon occurs due to the domination of entropic over enthalpic effects as the temperature increases above the LCST [30,42]. Below the LCST, PNIPAm chains exist in an extended coil conformation, and solvation is driven by the enthalpic gain from intermolecular hydrogen bonding between the PNIPAm chains and water molecules [16,49]. Solvation is further encouraged by a type of hydrophobic hydration, where the water molecules surround the a-polar isopropyl entities in a cage-like structure [50]. As the temperature is increased towards the LCST, intramolecular hydrogen bonding between carboxyl and amide groups on the PNIPAm chains result in the interruption of hydrogen bonding of these groups with water molecules, ultimately resulting in the chain adopting a collapsed conformation, driving out the water, and causing the polymer to precipitate out of solution [49]. These properties of PNIPAm and its copolymers make them applicable to a diverse range of pharmaceutical and biomedical applications [5,13,51,52].

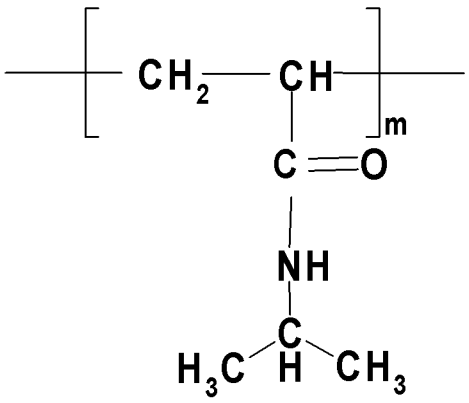

Figure 3: Chemical structure of PNIPAm [22].

The thermoresponsive behavior of PNIPAm may be altered by the introduction of hydrophobic or hydrophilic groups into the polymer structure. By copolymerization with more hydrophobic monomers, like N-tBAAm (N-tert-butylacrylamide), the LCST of the polymer is shifted to lower temperatures while copolymerization with more hydrophilic monomers, like AAm (acrylamide), increases the LCST [26]. Rochev and co-workers have synthesized a series of copolymers based on N-isopropylacrylamide (NIPAm) and N-tBAAm. Increasing the amount of the hydrophobic monomer (from a mole ratio of 0 to 50 %) increases hydrophobicity and therefore lowers the LCST (from 33 to ~10 °C) [53]. It was also observed that cell adhesion, cell growth properties and drug elution from cast NIPAm/N-tBAAm copolymer films were all dependent on the copolymer

composition [54,55]. In contrast, by increasing the amount of a hydrophilic monomer AAm in the NIPAm/AAm copolymers (from a molar ratio of 100:0 to 75:25), the LCST is increased (from 33 to 47 °C) [56].

4 POLYMER CHARACTERISATION BY FLUORESCENCE

The use of fluorescence spectroscopy for the evaluation of thermoresponsive polymers can provide useful insights into both the gross physical-dynamic processes and the more subtle physicochemical changes that occur in these polymer systems. Fluorescence spectroscopy offers high sensitivity for low probe loading and fast response times, thus minimizing perturbation of the polymer system. In thermoresponsive polymers like PNIPAm, the most common application of fluorescence measurements are for the study of micelle formation, aggregation dynamics, and the major phase changes that occur at the LCST. However, fluorophore choice needs to be very carefully considered because multiple factors (Figure 4) are at play and the environment is considerably more complex than that encountered in solvents. This potential multi-factor environmental sensitivity may mean that the observed changes in absorption / fluorescence intensity, shifts in absorption / fluorescence spectra, anisotropy, or fluorescence lifetimes originate from a combination of effects [57-59]. For example, when studying the phase change behavior of thermoresponsive polymers one needs to ensure that the selected fluorophore does not have a significant intrinsic temperature dependence, which may be convoluted with the responses due to the temperature, induced changes in polymer conformation.

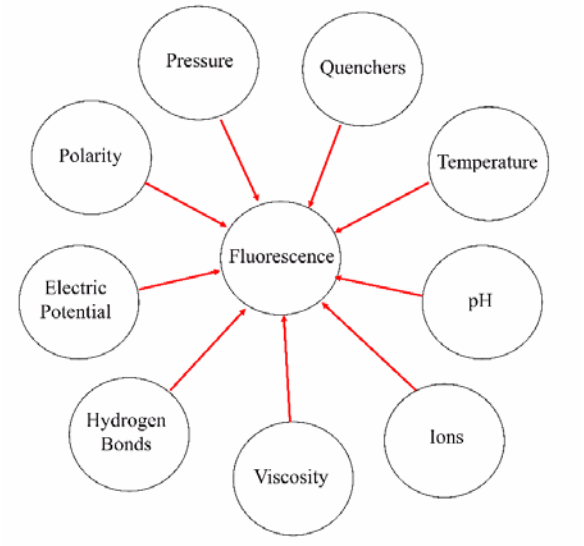

Figure 4: Some of the environmental factors which may affect impact on emission properties of fluorophores in polymers [60].

Another very significant environmental factor to consider when dealing with bulk or thin films of thermoresponsive polymers is the issue of water uptake. Many thermoresponsive polymers like PNIPAm are appreciably hydrophilic because of the presence of polar amide groups and will absorb water from the atmosphere [61]. Thus in many cases where one wishes to study the behavior of thermoresponsive polymer thin films one will need to control, or account for the presence of water in the thin films [62].

The potential range of fluorophores available for the analysis of thermoresponsive polymers is vast and a detailed analysis is outside the scope of this article. However, once a fluorophore has been selected there are only two general modes of employment: covalent attachment of the probe to the polymer [63], or deployment as a freely diffusing probe which is then introduced to polymer solutions or doped into thin films of the polymer. There are several drawbacks to the use of freely diffusing fluorophores ranging from the fact that they are free to diffuse out of the polymer structures, that they may aggregate, and that there is no control over where the probe interacts with the polymer. This obviously limits the potential applications; however, the approach is intrinsically very simple and lessens the risk of modifying polymer structure. One can minimize/eliminate many of these issues by covalently attaching the probe to the polymer; however, this may not always be feasible or straightforward. One critical aspect of the covalent labeling approach is to decide if it is possible to introduce the fluorophore during or after the polymerization process. For characterization of thermoresponsive polymers there are a wide range of covalently labeled fluorophores described in the literature. Some examples include pyrene and napthalene [63-66], *N,N*-(dimethy1amino)naphthalenesulfonamide (dansyl) [67], and the cyanine Cy5/Cy5.5 pair for FRET studies [68].

5 THERMORESPONSIVE POLYMER CHARACTERIZATION.

The fluorescence analysis of thermoresponsive polymer systems can be categorized, for the sake of textual organization, as either physiochemical or physical characterization. We note that there is a considerable degree of overlap and that in practice these factors should never be considered in isolation. The chemistry influences the physical factors and *vice-versa*. Here, we begin with physiochemical characterization and the crucial factor of water absorption, which then leads to the measurement of the chemical and polarity properties of thermoresponsive polymers. For the physical characterization, we start with Critical Micelle Concentration (CMC) and aggregation measurements and then progress to the study of phase transitions around the LCST, before finally showing how fluorescence can be utilized to study the assembly/behavior of thermoresponsive particles and surfaces.

When undertaking a physicochemical characterization of thermoresponsive polymers we have to consider first the chemical structure of the polymer and second the environment created by the polymer

when it is in solution or deployed as a particle or a thin film. In the first case Hydrogen-bonding, van der Waals interactions, and conformational changes are all significant, particularly in solution. In the second case where the polymer is fabricated into a higher density form, all these factors are again very important, but one must also take into account solvent/water infiltration which can mediate the chemical behavior of the polymer very significantly.

5.1 WATER SORPTION

Water uptake in a thin polymer film can lead to significant changes in the physicochemical properties of the polymer [69,70]. In critical applications like medical devices, this may lead to such problems as reduced adhesion and mechanical properties, pronounced physical and chemical aging, and swelling and expansion, compromising the intended function of the polymer and also modulating biocompatibility. The situation will be exacerbated when using thin films because the surface to mass ratio is much larger which facilitates water uptake. One of the most important considerations with thermoresponsive polymers is that they can be appreciably hygroscopic above and below the LCST. For PNIPAm, thermal gravimetric measurements on bulk polymer, indicated that no water was adsorbed at 40 °C (or more correctly, it was not possible to measure the low amount of adsorbed water) [71]. However, when PNIPAm is fabricated as a thin film then one can observe significant water absorption both below and above the LCST. For instance, a 10 μm thick PNIPAm film in an environment with a relative humidity of 90 %RH, was measured to have absorbed 26.5 % by weight of water below the LCST (at 25 °C). Above the LCST (at 37 °C) the amount of adsorbed/absorbed water was still a very significant 6.1 % by weight [61]. While the water absorption can be described as a purely physical effect, it has consequences for the chemical properties of the polymer because it affects the integral hydrogen bonding within the polymer. Thus for the relatively hydrophilic polymers like PNIPAm in thin film form, there is a clear requirement to handle the polymers under conditions of controlled humidity.

Fluorescent 3-hydroxyflavone (3-HF) derivatives have been used to monitor the infiltration of water in PNIPAm thin films [61]. 3-HF emission is governed by an excited-state intramolecular proton transfer (ESIPT) process and these probes demonstrate very strong solvatochromism and electrochromism [72-74]. These 3-HF fluorophores thus exhibit dual band fluorescence emission which is sensitive to environmental factors, and as a sensor/probe they have a clear advantage over single band fluorophores [75-80]. One of these bands originates from the normal excited state (N*), and the other is due to the ESIPT reaction product tautomer (T*). This emission, in terms of the wavelengths of maximum emission and relative intensities of the two emission bands, is sensitive to various environmental factors [72-74,81-83]. The most important emission parameter is the ratio of the emission intensities from the N* and T* excited states, I_{N^*}/I_{T^*}, which is associated with the relative populations of the N* and T* states and is a very sensitive indicator of solvent polarity [82]. The behavior of these emission bands as well as the relationship of their intensities depends strongly on

probe structure. Changing the chemical structure at the fluorophore core can be used to adjust the sensitivity to a specific range of solvent polarity, to modulate hydrogen bonding, or to electric fields. 4'-Diethylamino-3-hydroxyflavone (FE), 5, 6-benzo-4'-diethylamino-3- hydroxyflavone (BFE), and 4′-diethylamino-3- hydroxy-7-methoxyflavone (MFE) are examples of 3-HF fluorophores, which are sensitive to the properties of solvent environment. An increase in solvent polarity and hydrogen bonding ability of the solvent leads to an increase in the population of the N* form relative to T* form, which is due to a greater dielectric stabilization of the N* form [74]. It has been possible to correlate the ratio of the emission intensities of these two forms with changes in polarity and hydrogen bonding in various classes of solvents [74,84]. BFE, unlike FE and MFE, is nearly insensitive to the H-bond donor ability of protic solvents because of the interference from the additional benzene ring [84]. MFE shows more solvent-dependent dual emission in more polar solvents when compared to FE [77].

For these three 3-HF probes (FE, MFE, and BFE) it was observed that the relative emission from the N* and T* bands (as measured by the increase in $\log(I_{N^*}/I_{T^*})$) varied very significantly with water ingress (Figure 5). The N* band was also significantly red shifted whereas the T* band maximum decreased only slightly with increasing humidity. Analysis of the fluorescence data indicated that water adsorption in the PNIPAm films followed a two-step process: First, at low relative humidity the incoming water molecules disrupted the specific polymer-fluorophore H-bond interaction giving rise to small changes in $\log(I_{N^*}/I_{T^*})$ ratios and the overall fluorescence intensity. Then at higher relative humidity (>50%), as the amount of adsorbed water increases, these intensity parameters change dramatically indicating a larger change in the local polarity of the probe environment [61].

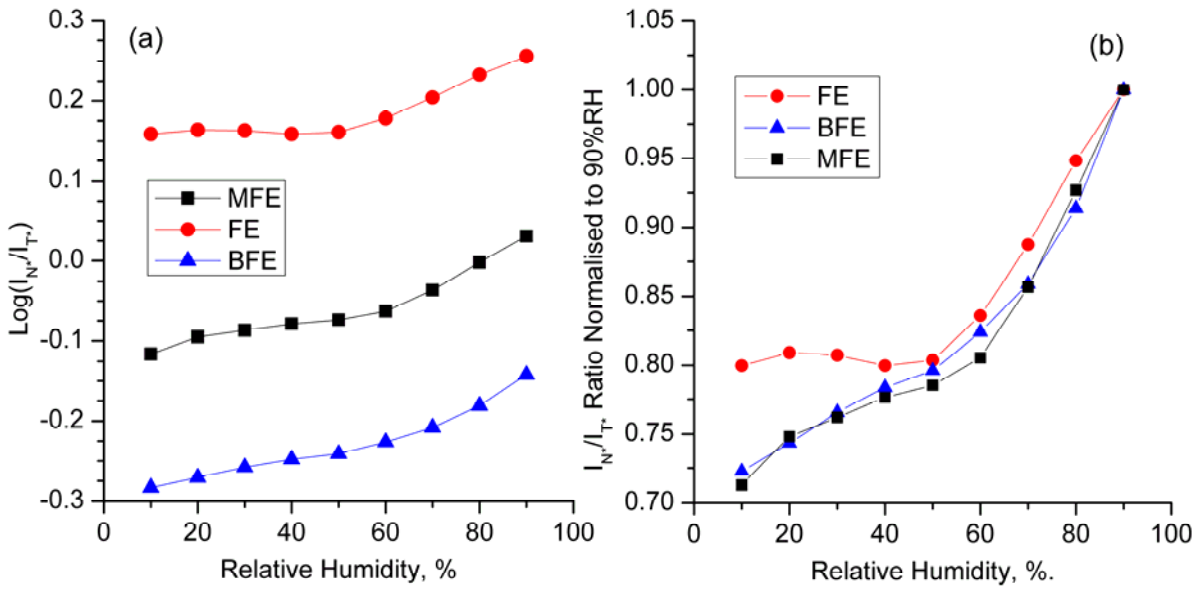

Figure 5: (a) Plot of the $\log(I_{N^*}/I_{T^*})$ ratio vs. increasing relative humidity at 25 °C for PNIPAm films doped with BFE (▲), MFE (■), and FE (●). (b) Plot of the I_{N^*}/I_{T^*} ratio normalized to the value recorded at 90% RH. Plots show the two-phase water adsorption process. Taken from ref [61] and reproduced with permission.

Emission measurements carried out above the LCST showed similar log(I_{N*}/I_{T*}) changes, except that the magnitude was lower. This indicates that the PNIPAm films were still absorbing some water in this more hydrophobic state, interrupting the polymer-probe interaction (Figure 6).

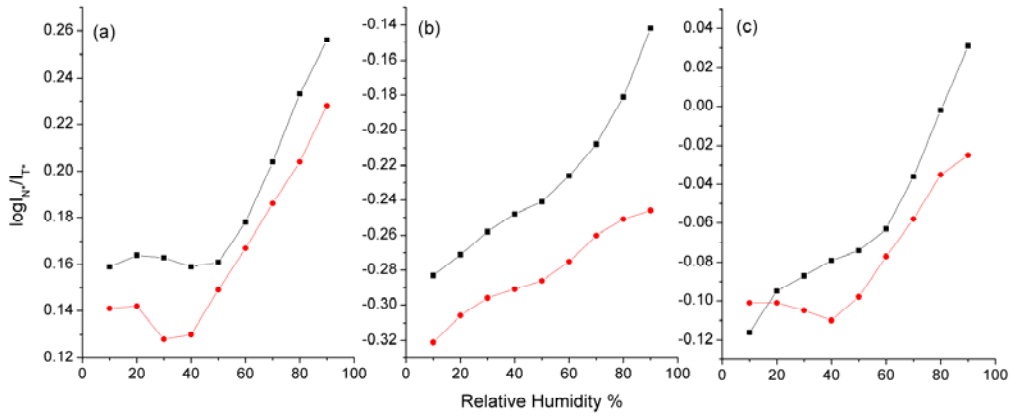

Figure 6: Plot of log(I_{N*}/I_{T*}) ratio vs. increasing humidity of PNIPAm films doped with (a) FE, (b) BFE and (c) MFE at 25°C (■) and 37°C (●). Taken from ref [61] and reproduced with permission. *Copyright © 2010 American Chemical Society, all rights reserved.*

In the more hydrophobic, condensed state above the LCST one would have expected that the rate of water adsorption should be decreased. However, fluorescence analysis of PNIPAm thin films show clearly that in every case, the *log(I_{N*}/I_{T*})* value increases at a similar rate (Figure 6). This method thus provides a relatively facile, non-contact methodology for assessing the ingress of water into thermoresponsive polymers when fabricated as thin films on solid surfaces.

5.2 PHYSICOCHEMICAL PARAMETERS BY SOLVATOCHROMISM

Measuring polarity and hydrogen bonding changes in thermoresponsive polymers is vital for understanding how these polymers will behave in the real world. For example, we know that many of the common thermoresponsive polymers are appreciably hygroscopic and will absorb appreciable amounts of water below and above the LCST thus influencing polarity within thin films [61]. The most widely based methods for assessing polarity is based on solvatochromism, and there is an extensive literature on solvent systems. Solvatochromism describes the change in shape, location and sometimes intensity of absorption or emission spectra, relative to the polarity of the medium. A hypsochromic (or blue) shift corresponds to negative solvatochromism, while a bathochromic (or red) shift corresponds to positive solvatochromism with increasing solvent polarity [85-87]. Solvatochromic methods are generally robust and reasonably easy to implement but do require the use

of relatively high probe concentrations compared to fluorophore indicators [88]. The most common solvatochromic solvent polarity scales are the: $E_T(30)$ scale of Dimroth and Reichardt [85], α and β scales of Kamlet and Taft [89,90], π* scale of Kamlet, Abboud, and Taft [91], and the pyrene (Py) scale of Dong and Winnik [92]. These solvatochromic methods are typically used to characterize solvent systems but they have also been extended to study polymers.

The $E_T(30)$ scale uses the 2, 6-diphenyl- 4- (2,4,6- triphenyl- 1-pyridino)- phenolate betaine dye (also known as Reichardt's dye) [85,93]. The $E_T(30)$ polarity parameter is based on the transition energy for the longest wavelength absorption band measured in the relevant environment and expressed in Kcal·mol^{-1} [85]. A normalized scale (E_T^N) was defined from 0 to 1 using tetramethylsilane and water as the extreme nonpolar and polar solvents respectively [85,93]. The α and β scales provide a measurement of solvent hydrogen-bond donor (HBD) or a hydrogen-bond acceptor (HBA) ability. These scales are based on a solvatochromic comparison method (SCM) which involves the comparison of solvent induced shifts of the longest wavelength absorption band of two similar compounds. For the determination of α values one compound is capable of acting as a HBA towards HBD solvents (*e.g*. Reichardt's dye) whereas the other cannot (*e.g.* 4-nitroanisole) [89,90,93]. To determine β values, one compound will be capable of acting as a HBD towards solvents (*e.g*. 4-nitroaniline) whereas the other cannot (*e.g*. 4-nitro-N,N-dimethylaniline) [93]. The solvent dipolarity/polarizability π* scale provides a quantitative measure of the non-specific part of van der Waals interactions between solvents and solutes [91,94,95]. The original scale was based on the spectral properties of carefully selected aromatic molecules which contain both electron-acceptor and electron-donor groups [94,95]. Dimethyl sulfoxide (DMSO) and cyclohexane (c-C_6H_{12}) were used as reference solvents by taking π*(c-C_6H_{12}) = 0 and π* (DMSO) = 1 [94,95]. More recently, the scale has been updated/revised and is now based on the averaging of data from several solvatochromic indicators [96]. Laurence *et al.* re-determined π* values for 229 solvents using only two solvatochromic indicators, 4-nitroanisole and N,N-dimethylamino-4-nitroaniline [85,96].

For the physicochemical characterization of polymers by optical spectroscopy, one generally uses either vibrational or electronic spectroscopies. One of the key advantages of electronic spectroscopy (either absorption or fluorescence) is the potential sensitivity, enabling the observation of subtle effects in condensed media. One of the simplest approaches is to pursue a solvatochromic approach and measure the UV-visible spectra of the appropriate indicators doped into the polymers [97-99]. For example, Matsuguchi *et al.* employed solvatochromic methods to characterize the water sorption behavior in polymer films [99]. They found that indicator band positions (and thus the solvatochromic parameters) were dependent on the polymer type, molecular weight, and the amount of absorbed water. On increasing relative water vapor pressures, all the solvatochromic parameters (α, β, π*) were seen to increase except for EC, PEO, PVP where the β values were lower at the higher water vapor pressures. The authors also examined the relationship between the $E_T(30)$ scale and the π*, α and β parameters and a linear correlation was observed indicating that the microenvironments experienced by the probe molecules in both wet and dry films was similar to that observed in liquid

solvents.

Szczupak *et al.* evaluated the micro-polarity and H-bond donor/acceptor ability for a series of thermoresponsive N-isopropylacrylamide/N-tert-butylacrylamide (NIPAm/NtBA) copolymer films using the $E_T(30)$, α, β and π^* empirical solvatochromic parameters [19,60]. For the dry NIPAm/NtBA copolymer films it was found that they are strong H-bond acceptors (β), moderate H-bond donors (α) and are strongly dipolar/polarizable (π^*). It was observed that $E_T(30)$, α and π^* values all decreased linearly on increasing the hydrophobic NtBA fraction in the copolymer films whereas the β parameter remained relatively unchanged. A good correlation was also found between experimental $E_T(30)$ and independently determined α, β and π^* values which confirms that the behavior of the solvatochromic indicators in the NIPAm/NtBA films was similar to that in solvents [19].

5.3 PHYSICOCHEMICAL PARAMETERS BY FLUORESCENCE:

While the application of solvatochromic methods for the characterization of physicochemical properties is feasible, and can generate accurate data, there is still a need for a more flexible measurement methodology. Specifically, for many applications, the concentrations required of the solvatochromic probes for absorption spectroscopy in these condensed media is relatively high (typical weight ratios of 36–20 to 1) [99], and it would be preferable to utilize much lower concentrations such as the levels employed in fluorescence spectroscopy (typical weight ratios of 1900–2230 to 1) [100]. Another need is the requirement for standoff measurements in sealed environments and in such cases, fluorescence may be the only feasible option.

Pyrene is a common fluorophore used for polarity assessment because the intensities of various vibronic bands are very sensitive to solvent polarity [101,102]. Karpovich and Blanchard explained that the relative changes in vibronic band intensities of the pyrene fluorescence spectrum are a result of vibronic coupling between the first (weakly allowed) and the second (strongly allowed) excited singlet states [103]. Their observations also suggest that solvent-dipole-solute induced dipole interactions play a major role in the pyrene scale. The extent to which an induced dipole moment is generated by vibrational distortions of pyrene is governed by the polarity of the solvent. The polarity of the pyrene environment may thus be estimated by measuring the ratio of the fluorescence intensities of the third and first vibronic bands (I_1/I_3) [101,102]. Dong and Winnik developed the Py scale of solvent polarities, based on this ratio and the scale is relatively insensitive to the hydrogen bonding ability of protic solvents [92]. Py scale values range from 0.58 for n-hexane to 1.95 for dimethyl sulfoxide. Variations in the pyrene lifetime can also be used to monitor changes in thermoresponsive polymers. For aqueous solutions of PNIPAm one observes approximately a 60 ns increase in average lifetime from 115 ns to ~175 ns as the temperature increases from 30 to 40 °C [104]. Szczupak *et al.* assessed the polarity of poly(NIPAm-co-NtBA) copolymer films by means of fluorescence based methods, using pyrene and 3-HF derivatives as polarity sensitive fluorescent probes

[100]. They reported a decrease in the I_1/I_3 ratio of pyrene with increasing NtBA fraction indicating a reduction in polarity with increasing NtBA content (Figure 7), which is in agreement with previous results obtained in a solvatochromic study involving the same polymers [19,100].

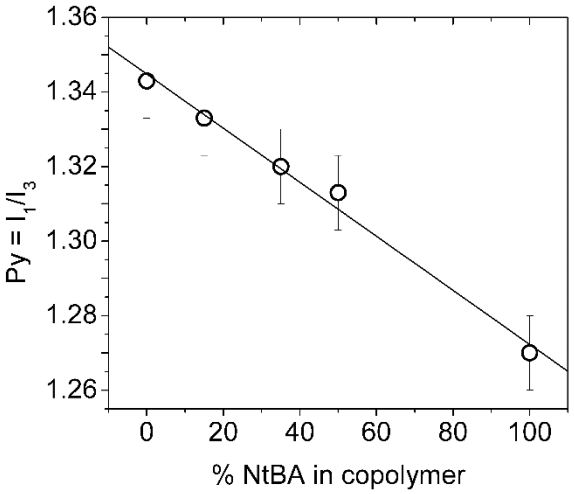

Figure 7: Plot of pyrene emission ratio I_1/I_3 ratio *vs*. NtBA content for a series of copolymer films measured at 20°C. Taken from ref [100] and reproduced with permission. *Copyright © 2010 Journal of Fluorescence Spectroscopy*.

The pyrene I_1/I_3 ratio displayed poor correlation with measured solvatochromic polarity parameters [19], which was attributed to the fact that the polarity component sensed by pyrene is only related to dipole-induced dipole interactions. This ignores the very significant H-bonding effects, which are also present in these polymers. Furthermore, it was noted that the physiochemical changes that occur at the LCST of these copolymers did not greatly affect pyrene fluorescence. This was revealed by the small and linear decrease in the I_1/I_3 ratio (1.345 to 1.285) observed for these thin films as the temperature changed from 20 to 40 °C. This contrasts strongly with the situation reported for aqueous solutions of PNIPAm where the ratio changes decreases from 1.79 to 1.38 [105]. We also note that this magnitude of change is almost identical to that observed for solutions of pyrene in ethanol and 1-propanol [60]. The reason why the change is small originates from the lack of a distinct aqueous phase, so the pyrene probe remains dispersed throughout the solid polymer where there is much less change in probe environment and thus in emission properties. It should be noted that these measurements were made without rigorous humidity control [100], and so these films probably contained a significant amount of adsorbed water. The hydrogen-bonding effects inherent in the PNIPAm thin films also produces problems for the use of 3-HF derivatives for polarity assessment of thermoresponsive hydrophilic polymers in thin films, using simple emission parameters. This arises from the heterogeneity of the ground-state H-bonding and an ESIPT process that is not in equilibrium,

both effects can be elucidated from the observed excitation wavelength dependence and a difference in the fluorescence lifetimes of the N* and T* bands [74,100,106].

One example where fluorescence was used to assess the polarity change at temperatures near the LCST exploited the solvatochromic shifts of the zwitterionic form of rhodamine X [107]. The authors employed a rhodamine X labeled oligonucleotide composed of 25-mers of thymine (dT_{25}-ROX). In dioxane/water mixtures this probe shows a linear relationship between $E_T(30)$ and shifts in the fluorescence emission maximum (in wavenumbers) for the $E_T(30)$ range between 50 and 65 $Kcal.mol^{-1}$. They used this probe to look at the polarity changes of the PNIPAm shell of PMMA/PNIPAm core-shell latex particles at temperatures near the LCST. For this system, the calculated $E_T(30)$ value for the PNIPAm shell decreases in a sigmoidal manner as the temperature increases from 15 to 45 °C. At the lower temperatures, the PNIPAm shell polarity is nearly identical to that of water, while above the transition it is equivalent to the polarity of a dioxane/water mixture (30% (v/v)). In contrast to the smooth transition observed in the wavelength shifts, the lifetime of the dT_{25}-ROX probe showed a sharp drop at the LCST which is ascribed to the refractive index change, which accompanies the dehydration process during the phase change.

Derivatives of quinoxaline (N-(2,3-dimorpholinoquinoxalin-6-yl)acrylamide, *QxA* and N-(1-(2,3-dimorpholinoquinoxalin-6-ylamino)prop-2-yl)methacrylamide, *QxAlaMA*) have also been incorporated into PNIPAm [108]. These fluorophores when incorporated into the polymer showed intense solvatochromism in their fluorescence without perturbing the LCST. The wavelength at the maximum fluorescence intensity of the *QxAlaMA*-labeled PNIPAm dramatically blue-shifted (by ~20 nm) and the fluorescence intensity of the *QxA*-labeled PNIPAm significantly increased, by a factor of ~10, as the temperature increased from 30 to 34 °C.

5.4 CRITICAL MICELLE CONCENTRATION (CMC) AND AGGREGATION MEASUREMENTS.

The CMC is a critical parameter that affects the macroscopic behavior of these thermoresponsive polymers. Measuring the CMC is thus an important facet of polymer science and can usually be achieved by conventional means using techniques like Dynamic Light Scattering (DLS). However, in some cases the CMC can be low and as such is not very amenable to conventional measurement methods. For example, the CMC of some amphiphilic block copolymers is of the order of $\sim 10^{-6}$ M and cannot be easily determined by scattering methods. This problem can be overcome by using single molecule detection (SMD) methods like fluorescence correlation spectroscopy (FCS) where one can easily observe the very low concentration regime [109-111]. One straightforward method collects FCS data from samples which have a fixed, very low concentration (typically 50 nM or less) of a poorly water soluble fluorophore such as R6G in aqueous solutions of the thermoresponsive polymer with varying concentrations (micro-molar range). The polymer concentrations need to be

selected such that they span a range above and below the suspected CMC value. At polymer concentrations below the CMC, the fluorophore should remain dissolved in water, and only the diffusion of free dye should be observed (free R6G has a hydrodynamic radius of 0.8 nm) [112]. As the polymer concentration increase above the CMC value, an increasingly significant fraction of the fluorophores will become associated with the micelles as they are formed. This association process should result in the observation of an additional slow diffusion process being incorporated in the FCS correlation curve.

For example, Adelselberger and co-workers used FCS to study the behavior of amphiphilic, symmetric tri-block thermoresponsive copolymers having short, deuterated polystyrene (PS) end blocks and a large PNIPAm middle block in aqueous solutions at very low concentration close to the CMC [111]. Using FCS they found that at polymer concentrations above 0.9 μM, a second, slower decay appeared in the correlation curves, indicating the onset of micelle formation. Fitting of the correlation curves yielded hydrodynamic radii of 20.8 ± 0.7 nm for PS_{11}-b-$PNIPAm_{280}$- b-PS_{11} and 25.8 ± 0.9 nm for PS_{11}-b-$PNIPAm_{370}$-- b-PS_{11}. These values were validated from DLS measurements made at dilute (0.1 mg/mL) concentrations. The authors do however, point out that with these free probes, micelles may still be present at very low concentrations (< 0.9 μM), but that there may be too few to solubilize ample Rh6G molecules to generate sufficient signal that can be extracted from the correlation curves. A variation of this methodology is to use polymer covalently labeled with a fluorophore and measure the change in hydrodynamic radii via FCS [109,110]. This can be a very sensitive method for determining the hydrodynamic radii of not only the micelles but also the unimers, and intermediate, unstable aggregates.

While these SMD methods are elegant, care needs to be taken with interpretation of results because of complications induced by the use of very low fluorophore and polymer concentrations. These include changes in polymer and probe concentrations due to surface binding to the container walls, which can be different for the free polymer and the various polymer assemblies. Consideration also needs to be given to variations in fluorophore photophysics (quantum yield, lifetime *etc*.) generated by the different physical environments of the solution and the micelle [113].

An extension of the FCS methodology was used by Wang and co-workers to study lateral diffusion on PNIPAm brushes attached to solid surfaces [114]. They used a poly(2-vinylpyridine) probe molecule that was covalently labeled with Alexa 488. Their studies enable the extraction of friction force data and also a better understanding of how the PNIPAm brushes interact with polyelectrolyte probe molecules. They showed that below the LCST, the decrease of viscosity of the solvent water brought about a decrease in the friction forces via coupling of the lateral probe diffusion with the motion of the brush chain. The LCST transition induced stiffening of the PNIPAm chain and the hardening of the PNIPAm brushes above the LCST generated a large increase in friction forces.

Another study compared the diffusion of probe molecules interacting with octadecyltriethoxysilane (OTE) monolayers and surface-tethered PNIPAm polymer chains of varying thickness and surface coverage [115]. An interesting observation from this study was that the R6G fluorophore interacted more strongly with PNIPAm compared to the OTE monolayer. This indicates

that the coupling of interfacial interaction and polymer chain dynamics causes a greater slowing of probe diffusion. The sensitivity of the FCS methodology is very beneficial here because it allows the differentiation of many different effects.

Diffusion of large particles formed from thermoresponsive polymers has also been studied using more conventional fluorescence microscopy techniques like Fluorescence Recovery after Photobleaching (FRAP). In the FRAP technique the sample is observed under a microscope (usually a confocal laser scanning microscope), a region of interest (RoI) is rapidly bleached using relatively high power excitation, then the region is imaged over time to determine how long it takes for new fluorescent molecules/particles to diffuse back into the RoI. Relatively simple image analysis can be used then to recover useful information such as diffusion rates, which in turn can be used to ascertain changes in particle size. For thermoresponsive polymers one can use the FRAP method to observe and measure physical effects on the micro to macro size scale. One example used FRAP to quantify the variation in permeability of the walls of thermoresponsive hollow capsules with temperature [116]. The two di-block copolymers of PNIPAm studied were prepared as hollow capsules, and using standard confocal laser scanning microscopy one could monitor temperature induced changes in particle size. The permeability of the thermoresponsive shells could be assessed by comparing the degree of infiltration of two differently sized fluorophores, 6-carboxyfluorescein and fluorescein-labeled dextran.

ANS (1-anilino-8-naphthalene sulfonate) is one of the most widely used polarity probes because it is highly fluorescent in low polarity solvents but is weakly fluorescent in aqueous solution [101,117]. This important feature enables one to visualize the hydrophobic regions of a given system with minimal influence from ANS molecules remaining in the aqueous environment, and thus this fluorophore has found widespread application in biological and material sciences [118-123]. Kujawa *et al.* utilized ANS to study the concentration (0.02 to 10 g/L range) and temperature dependent solution properties of telechelic PNIPAm (C_{18}-PNIPAM-C_{18}) [124]. As polymer concentration was increased a steep increase in emission intensity of ANS was observed, accompanied by a blue shift of emission band maxima. These observations with increasing polymer concentration indicate the increased hydrophobicity of the probe (ANS) environment (Figure 8).

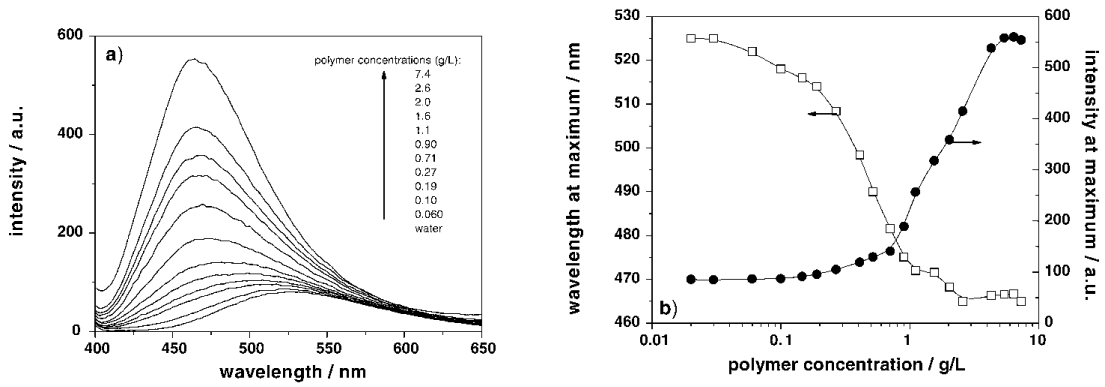

Figure 8: (a) Emission spectra of ANS in water and in aqueous solution of telechelic PNIPAm at 20°C. (b)

Fluorescence intensity and position of emission band maxima as a function of polymer concentration at 20°C. Reproduced with permission from ref [124]. *Copyright © 2005, Springer Berlin / Heidelberg*.

These fluorescence results support the results from DLS that suggest for the telechelic PNIPAm in solution, the degree of aggregation within the rosettes increases as the concentration increases, and that the steric crowding results in expulsion/release of polymer bound water in order to accommodate the increased steric pressure.

5.5 PHASE TRANSITIONS AT THE LCST.

The phase transitions that occur at the LCST are the defining feature of thermoresponsive polymers. While the phase transition is often easily observed (with high concentration solutions or when the polymer has been fabricated into a film), there are cases where the use of sensitive fluorescence methods are warranted. This is particularly the case where the polymer concentration is low, or where a non-contact method is required. Both the simple freely diffusing probe and the more complex covalently bound probe approaches have been successfully employed. Winnik utilized pyrene labeled PNIPAm to study the temperature induced phase transitions in aqueous solutions [105,125]. One study showed that the complex pyrene photophysics when covalently labeled with PNIPAm was dependent on the degree of labeling and involved emission from monomers and excimers [105]. At ambient temperature in water, the presence of ground-state pyrene dimers and higher aggregates was observed for the pyrene labeled PNIPAm. These aggregates can form between pyrene attached to the same chain or between pyrene probes located on different chains. Heating solutions of the labeled PNIPAm above the LCST results in disruption of the pyrene aggregates and the quantum yield of the monomeric species increases relative to that of the excimers. This dissociation is complete in the case of the sparsely labeled polymer(PNIPAm/Py/200) but only partial in solutions of the more highly labeled polymer (PNIPAm/Py/20). When trace amounts of the labeled PNIPAm were added to solutions of unlabeled PNIPAm, changes in pyrene fluorescence could be used to ascertain the interaction between chains. They found that below the LCST, there was no indication of interactions between labeled and unlabeled polymers, whereas above the LCST, the labeled polymers were incorporated into the PNIPAm-rich phase. These fluorescence studies also showed that in the low concentration limit (<1 ppm) for highly labeled polymers there was evidence for the formation of single-polymer chain micelles. More recently, pyrene was employed to study long-range polymer chain dynamics for PNIPAm using a variety of models including the Fluorescence Blob Model (FBM) [126,127]. Chee *et al.* have also investigated in detail the interactions between PNIPAm and pyrene using time resolved fluorescence spectroscopy and concluded that above the LCST PNIPAm is capable of solubilizing hydrophobic guests such as pyrene but that below the LCST much of this capability is lost [128].

A more facile method for assessing the effects of the phase changes in aqueous solutions of

telechelic PNIPAm (C_{18}-PNIPAM-C_{18}) involved the use of ANS and the measurement of steady-state emission spectra. When the emission was measured over the 10 to 50 °C temperature range one observed at approximately 29 °C a sharp increase in fluorescence intensity coupled with a blue shift in the emission band maximum (Figure 9). This was clear evidence for a very significant change in the micropolarity sensed by ANS, as with increasing temperature ANS passes from a hydrophilic environment of "*highly hydrated rosettes*", formed at lower temperatures to the "*hydrophobic medium of collapsed and associated polymeric micelles*" [124].

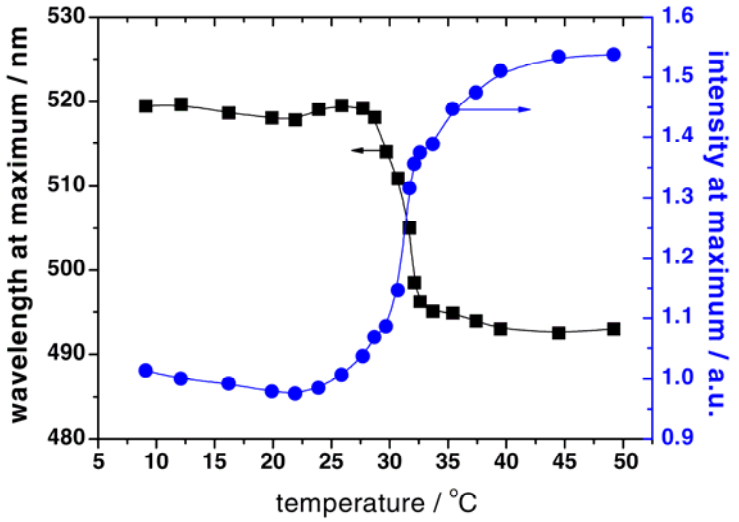

Figure 9: Temperature dependence of the ANS fluorescence intensity and wavelength of emission band maximum in polymer solution (polymer concentration is 0.1gL^{-1}). Reproduced with permission from ref [124]. *Copyright © 2005, Springer Berlin / Heidelberg*.

Another method for analyzing thermoresponsive polymer transitions near the LCST is to utilize polarization anisotropy in conjunction with fluorescence quenching studies. The use of acenaphthylene (ACE) covalently labeled PNIPAm for studying the effect of urea on polymer structure/dynamics in solution has been demonstrated several times [129,130]. ACE was used because there is no rotation independent of the segment to which the ACE probe is attached. Steady-state anisotropy studies showed that below the LCST, anisotropy was small irrespective of the presence of urea (a quencher), which was consistent to the open structure of the loose coil polymer conformation [130]. Above the LCST, large *anisotropy* values were recorded in the absence of the quencher indicating that the polymer had adopted a compact conformation. However, when urea was added, the anisotropy decreased very significantly indicating that the polymer conformation had opened up.

A combination of anisotropy and FRET has been used to probe the behavior of mesoglobular phases formed when PNIPAm was heated in solution [66]. Anisotropy measurements conducted on napthyl labeled PNIPAm gave an indication of the rotational freedom of the pendant fluorophore and this was related to the micro-viscosity of the phase in which it was dissolved. The results indicated

that solutions heated within the 31–36 °C temperature range consisted of fluid-like particles which were able to merge and grow in size. At higher temperatures (36–45 °C) the PNIPAm mesoglobules behaved like more rigid spheres that were unable to merge by collision into larger, slower rotating globules. This study also employed FRET measurements using pyrene (Py) and naphthalene (Np) labeled PNIPAm. Monitoring the changes in Py and Np fluorescence shows the expected spike in the I_{Py}/I_{Np} fluorescence intensity ratio near the LCST. The authors ascribed this change as arising from two factors: (a) chain motion increased upon heating, enhancing the probability of a close encounter between the two different fluorophores, and (ii) the local fluorophore concentration increased as a result of solution demixing. However, at temperatures above ~33.5 °C, the I_{Py}/I_{Np} fluorescence intensity ratio decreased once more which was ascribed to the increased micro-viscosity within the mesoglobule. This restricted the motion of the polymer chains, which caused a reduction in the frequency of the Py-Np encounters.

The study of Quantum dot (QD)-PNIPAm hybrid particles by FCS has also been recently reported [131]. This straightforward study described how FCS can be used to monitor changes in hydrodynamic radii near the LCST for particles having different length PNIPAm chains attached. It also indicated that there were substantial decreases in the QD lifetime as the temperature increased through the LCST caused by PNIPAm chain collapse. This alteration in average lifetimes was caused by large changes in the fast lifetime component, which is generally assigned to trapping processes caused by surface defects or impurities.

5.6 SWELLING AND ASSEMBLY.

Dansyl is a versatile probe for characterizing PNIPAm as it can provide information via steady-state and time-resolved measurements about the polarity and viscosity of the local environment [67,132,133]. Some of the advantages of the dansyl probe are that it is relatively insensitive to oxygen quenching, its absorption maximum is relatively independent of the medium and variation in the wavelength of maximum emission is directly related to changes in local polarity and/or viscosity.

The mechanism of shrinking in PNIPAm derived materials can be elucidated by measuring changes in the wavelength of the fluorescence emission maximum of a covalently attached dansyl fluorophore [133]. The rate of shrinkage of different PNIPAm gels in aqueous solution (normal, with grafted side chains, and semi-interpenetrating polymer network) was investigated by measuring the change in the position of the peak emission wavelength. These fluorescence measurements showed clearly that the grafted chains underwent the coil-to-globule transition at lower temperature (~25 °C) than the main polymer chains (~33 °C). Dansyl (covalently labeled) has also been used as a probe to look at the changes due to cross-linking PNIPAm [134]. In this case, cross-linking with glutaraldehyde caused an increase in hydrophobicity which could be evaluated by measuring the blue shift in the fluorescence emission spectra.

Dansyl labeled PNIPAm (PNIPAm-Da label content, 0.06 molar %) was also used to investigate

the phenomenon of cononsolvency exhibited by the PNIPAm/water/methanol ternary system. The study involved both the PNIPAm-Da polymer and the cross-linked PNIPAm-DA gels [67]. The swelling of these polymers and gels decreased abruptly in aqueous solutions containing 7-25 mol % methanol and increased gradually in systems with a higher methanol concentration. Shifts in the dansyl wavelength of maximum emission, changes in the fluorescence lifetimes, and changes in the rotational diffusion coefficients could all be correlated with the macroscopic changes in swelling volume.

In some cases, the swelling behavior of PNIPAm can be affected by the presence of other materials in solution. When freely diffusing pyrene was used as a probe of PNIPAm behavior, the introduction of urea changes in the pyrene emission only above the LCST [130]. This arose from urea induced swelling of the PNIPAm compact coil conformation, caused by disruption of the intramolecular hydrophobic interaction. This caused the pyrene probe to experience a much more hydrophobic environment which was measured by the ratio of the emission intensities I_3/I_1. This study also showed that the coil structure was reasonably robust and could be observed at urea concentrations of up to 3M.

The swelling behavior of thermoresponsive polymers can also be investigated using Förster Resonance Energy Transfer (FRET), where the degree of energy transfer provides information as to separation between donor and acceptor fluorophores [59]. For example, Jones and co-workers used FRET studies to analyze core-shell PNIPAm microgels (both the core and shell components were lightly cross-linked with *N,N′*-methylene(bisacrylamide)), where the core was doubly labeled with cyanine Cy5 (donor) and Cy5.5 (acceptor) [68]. In these structures, the PNIPAm shell can restrict the core from swelling to its native volume, and thus the extent of core expansion will be a function of shell thickness. To covalently attach the fluorophores, the core contained a small percentage of amine groups for post-polymerization modification with the cyanine fluorophores, which were functionalized with *N*-hydroxysuccinimidyl ester. For the naked core, the degree of FRET was low when it was swollen to its maximum volume below the LCST (31 °C). The presence of a PNIPAm shell produces a significant degree of FRET under the same solution conditions, indicating that the polymer chains are more constrained relative to the fully swollen state. By monitoring the degree of energy transfer in the absence and presence of the shell, over a range of temperature values, the researchers observed the decreased swelling ability of the core in the presence of the added shell, and were thus able to estimate the shell thickness. This FRET method has obvious advantages when compared to conventional Photon Correlation Spectroscopy (PCS) measurements, which can only yield an apparent particle size, and not discriminate between changes in shell thickness and core compression associated with thicker shells.

The combination of thermoresponsive polymers and the FRET methodology has also been exploited for sensing applications. In one such example, PNIPAm microgels were modified to incorporate potassium ion recognizing 4-acrylamidobenzo18-crown-6 residues (B18C6Am) and then a FRET pair of fluorophores (4-(2-acryloyloxyethylamino) -7-nitro-2,1,3-benzoxadiazole (NBDAE), and rhodamine-B-based FRET acceptors (RhBEA)) [135]. The key operational feature is the fact

that the polymer LCST is directly affected by the K^+ ion concentration, increasing by ~9 °C as the K^+ concentration increased from 0 to 300 μM. This reasonably rapid process (~4 second response time) can be monitored by measuring the FRET efficiency calculated from the fluorescence intensity ratio measured at 588 and 529 nm.

Many biomedical uses for PNIPAm involve the preparation of complex macro-, or meso-scale structures and the assembly process can be studied using fluorescence. For example, the 4-acrylamidofluorescein-modified poly(N-isopropylacrylamide-co-acrylic acid) (PNIPAm-co-AAc*) was used with simple fluorescence microscopy to observe layer-by-layer (LbL) deposition of microgel thin films [136]. The method is reasonably effective at showing coverage and layer formation, but because of its non-confocal nature the resolution normal to the surface is very poor. Another area in which fluorescence techniques can be useful for studying thermoresponsive polymers is in the LbL assembly of polyelectrolyte multilayers on soft and porous PNIPAm microgels. One facet of the LbL process is that the polyelectrolytes can interdigitate both with each other and with the microgel during multilayer formation. The problem is further compounded by the fact that the particles are often sub-micron in size and thus not amenable to conventional microscopy evaluation. Using FCS, however, one can easily distinguish between free, labeled polyelectrolytes and those that are bound to the microgel. Wong and co-workers used dual color FCS to confirm that two different polyelectrolytes were binding onto the same microgel particles (~400 nm in size) of PNIPAm [137].

One of the drawbacks with conventional FCS measurements is that one cannot generally get absolute diffusion coefficients from the data and thus one has to correlate with standards of known values. In dual-focus FCS (2*f*-FCS), one uses two focal volumes, which are a precisely known distance apart and generate an overlapping detection volume. This enables accurate and precise quantitative measurement of absolute diffusion coefficient values [138]. 2*f*-FCS measurements have been undertaken at different temperatures to determine the hydrodynamic radii of bare nanogels (p(NIPAM-co-AA-co-rhodamine)) and nanogels coated with various numbers of layers of polyelectrolytes [139]. These temperature dependent studies showed that the polyelectrolyte multilayer shell was still bound to the nanogel during the phase transition at the LCST.

6 CONCLUSIONS

Fluorescence spectroscopy offers a range of convenient methodologies for the analysis of thermoresponsive polymers. The most widespread application is for the monitoring of the phase and polarity changes at the LCST. The high sensitivity and low probe concentrations required ensures that the fluorescence analysis has a minimal impact on polymer structure or physical behavior. Furthermore, many of these analytical techniques can be performed using standard off-the-shelf, inexpensive fluorescence spectrometers. Of increasing importance is the use of single molecule detection methods to probe the dynamic processes that occur at very low polymer concentrations.

This can provide unique information and insights into intra- and inter-chain interactions, allowing one to first examine the growth of polymer aggregates and other higher order structures in solution.

7 ACKNOWLEDGEMENTS

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