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A spectroscopic study of the self-association and inter-molecular aggregation behaviour of pH-responsive poly(L-lysine *iso*-phthalamide)

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Abstract

The pH mediated intra-molecular association and inter-molecular aggregation of a range of amphiphilic poly(L-lysine *iso*-phthalamide) polymers have been investigated in aqueous solution over a range of pH values and concentrations. The desired functionality of these novel bioresponsive amphiphilic polymers was achieved by incorporating pendant hydrophilic carboxyl groups along the polymer backbone, via the L-lysine moiety, balanced by a degree of hydrophobicity introduced via the *iso*-phthaloyl moiety. Incorporation of low levels of bis-functional Cy3 (poly-Cy3) and/or Cy5 dye (poly-Cy3/5 or poly-Cy5) co-monomers in the responsive polymer backbone allowed detailed probing of the pH mediated hydrophobic association using a combination of optical spectroscopic techniques. Both steady-state fluorescence spectroscopy and fluorescence lifetime measurements of poly-Cy3 revealed a conformational transition at pH 4.5. Thus, below a critical pH the polymer collapsed into a compact globular structure (hypercoil) bringing the fluorophore molecules into close proximity with one another. This resulted in a dramatic reduction in fluorescence intensity and fluorescent lifetime in the single fluorophore systems (poly-Cy3) accompanied by a red shift in the maximum emission wavelength. Observed redshifts in the emission maxima and enhancements of fluorescent lifetimes with increasing polymer concentration suggested the formation of polymer aggregates. Fluorescence resonance energy transfer (FRET) was measured in mixtures of single fluorophore containing poly-Cy3 (donor) and poly-Cy5 (acceptor) and dual fluorophore containing poly-Cy3 (donor)/Cy5 (acceptor) in an effort to distinguish between intra-molecular versus inter-molecular association. The relevance of the results with respect to potential in vivo applications (drug delivery and biodiagnostics) is discussed.

Keywords: Responsive polymer; Fluorescence; Conformation

1. Introduction

Responsive polymers, capable of sensing and responding to regional changes in environment such as pH, temperature and concentrations of metabolites, have considerable promise in a wide range of biomedical and biotechnological applications, e.g. bioseparations, biocatalysts and as controlled drug delivery vehicles and imaging agents [1–3]. However, whilst the potential of water-soluble polymers (and polymeric assemblies) to mediate drug delivery is well appreciated [4], low transport across extra and intra-cellular plasma membranes of cells is a principal barrier to the effective intra-cellular delivery of macromolecules [5]. pH-responsive polymers have been investigated as membrane destabilising agents, mimicking the role of amphiphilic peptides on the surfaces of certain viruses [6] to efficiently release endocytosed macromolecules into the cytosol before they reach the lysosomes [3,7–9]. Appropriately designed, hydrophobically modified, weak polyacids (or polybases) change conformation in response to changes in environmental pH from expanded structures, dominated by electrostatic repulsions, to collapsed structures, stabilised by hydrophobic association [10,11]. Such agents can disrupt the endosomal bilayer under slightly acidic conditions, pH (5.5–6.5), but be non-lytic under physiological conditions at pH 7.4 [12–15]. In particular, the pH mediated conformational transition and membrane destabilising effects of poly(methacrylic acid) [16,17], poly(ethylacrylic acid) [13] and poly(propylacrylic acid) [18] and the ability to tailor the specific pH of collapse towards the pH of early endosomes (around pH 6.5) by modification of the hydrophobic moiety have been well documented and characterised [19].

Eccleston et al. [20,21] have demonstrated membrane lytic activity for a metabolite derived, responsive, biocompatible

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polymer, poly(L-lysine *iso*-phthalamide) and we have reported the synthesis of a series of responsive 'Polydyes' by conjugation of a bis-amine Cy3 fluorophore within the backbone at the polymer synthesis stage. The fluorophores acted as reporters on the state of the polymer conformation in aqueous solution, which was manipulated either with solution pH or tonicity [21]. Moreover, modification of the associative behaviour of the responsive system and the pH of onset and magnitude of the degree of membrane lysis through grafting with hydrophilic side chains has recently been demonstrated by Yue [22,23] and Chen [24]. The synthesis and a preliminary characterisation of these polymers have been discussed in previous publications [15,20,21]. Furthermore, a poly(L-lysine *iso*-phthalamide) FRET system demonstrating pH mediated energy transfer from co-polymerised Cy3 (donor) to Cy5 (acceptor) has recently been proposed as a molecular pH probe for biomedical imaging applications, in particular the optical imaging of solid tumours [25]. However, a detailed study of polymer conformational dynamics in response to environmental stimuli has yet to be reported. This paper seeks to address these issues through the use of a combination of optical techniques. An understanding of the solution behaviour and ensuing physiological response to specific microenvironmental conditions is critical for successful drug delivery and in vivo diagnostic applications based on these novel biopolymers.

pH Dependent association of hydrophobically modified polyelectrolytes can be studied using the non-radiative energy transfer (NRET) between suitable chromophore pairs immobilised onto various responsive polymer backbones [26–32]. The technique can be used to probe the conformation and degree of interactions between polymer chains containing suitable chromophore pairs. These are typically held apart by electrostatic repulsions along the backbone of charged polymers, but can be brought into close proximity due to hydrophobic association of modified weak polyelectrolytes upon partial neutralisation. In this article we present a detailed characterisation of the conformational dynamics and fluorescent behaviour of a novel, double fluorophore containing poly(L-lysine *co*-bis-carboxy-Cy3 *iso*-phthalamide *co*-bis-carboxy-Cy5) over a range of polymer concentrations and pH. The dual fluorophore system is compared with a mixture of single Cy3 or Cy5 containing polymers using a combination of advanced optical methods including steady-state fluorescence spectroscopy and fluorescence lifetime imaging microscopy (FLIM).

2. Materials and methods

2.1. Materials

Iso-phthaloyl chloride, potassium carbonate, methyl-2 butanone, dimethyl sulphate, oxaloyl chloride, sodium acetate, *N*,*N*′-diphenylformamidine and malonaldehyde bis(phenylimine) monohydrochloride were obtained from Aldrich. L-lysine methyl ester dihydrochloride and *p*-hydrazino benzoic acid were purchased from Lancaster. Pyridine, acetic anhydride and glacial acetic acid were purchased from Fisher. Dimethyl sulfoxide (DMSO) and acetone were dried using standard procedures. All other reagents were used directly as received.

2.2. Cyanine dye synthesis

The cyanine dye precursor 1-methyl-2,3,3-trimethyl-5-carboxyl-3-*H*-indolium methyl sulphate (1) was synthesised by a modified method of Lindsey et al. [33]. *p*-Hydrazino benzoic acid (15.5 g) and 30 mL methyl-2 butanone were refluxed in 100 mL 95% ethanol for 30 min and the yellow hydrozone salt recovered (up to 90% yield) by filtration following cooling to 0 °C after removal of approximately 100 mL of the solvent. The resulting hydrozone was ring closed according to the Fisher indole route by refluxing in glacial acetic acid under nitrogen for 4 h to give a red oil on removal of the solvent. Recrystallisation could not be achieved from ethylacetate/petroleum ether as reported in the literature but redissolution in warm acetic acid followed by precipitation into ice cold deionised water gave an orange powder which was recovered by filtration and dried in a vacuum oven at 50 °C overnight. The resulting yellowish powder had a characteristic UV adsorption peak at 275 nm in methanol similar to that reported for the desired 2,3,3-trimethyl-5-carboxy-3-*H*-indole. Subsequent reaction of the indole with dimethylsulphate under careful reflux followed by destruction of excess dimethyl sulphate with wet methanol, removal of the volatiles by rotary evaporation and trituration with toluene gave the quaternised 1-methyl-2,3,3-trimethyl-5-carboxyl-3-H-indolium methyl sulphate salt.

2.2.1. 1,1′,3,3,3′,3′-Hexamethyl-5,5′dicarboxyindocarbocyanine methylsulphate (bis carboxy Cy3)

2.6 g of (1) (8 mmol, 2 equiv.) and 0.8 g of *N*,*N*′ diphenylformamidine (4 mmol, 1 equiv.) were added to a 1 L Florentine flask containing 100 mL of acetic anhydride, 50 mL glacial acetic acid and 3.16 g of sodium acetate (38.5 mmol, 9.5 equiv.). The solution was refluxed under nitrogen for 3 h developing an intense red colour. The volatile components were removed by rotary evaporation to leave a viscous pearlesant red oil. The resulting oil was chromatographed on sephadex LH-20 using methanol as eluent giving a dark red solid.

2.2.2. 1,1′,3,3,3′,3′-Hexamethyl-5,5′dicarboxyindodicarbocyanine methylsulphate (bis carboxy Cy5)

6.5 g of (1) (20 mmol) were added to a 1 L round bottomed flask containing 150 mL acetic anhydride and 50 mL pyridine. Malonaldehyde bis(phenylimine) monohydrochloride (3.23 g, 10 mmol) was added and stirred for 2 h at room temperature. A blue colour began to develop almost immediately becoming intensely blue at the end of the reaction. The volatile components were removed by rotary evaporation and the residual viscous blue oil recrystallised three times from acetone/toluene (1:5) to give a metallic green solid.

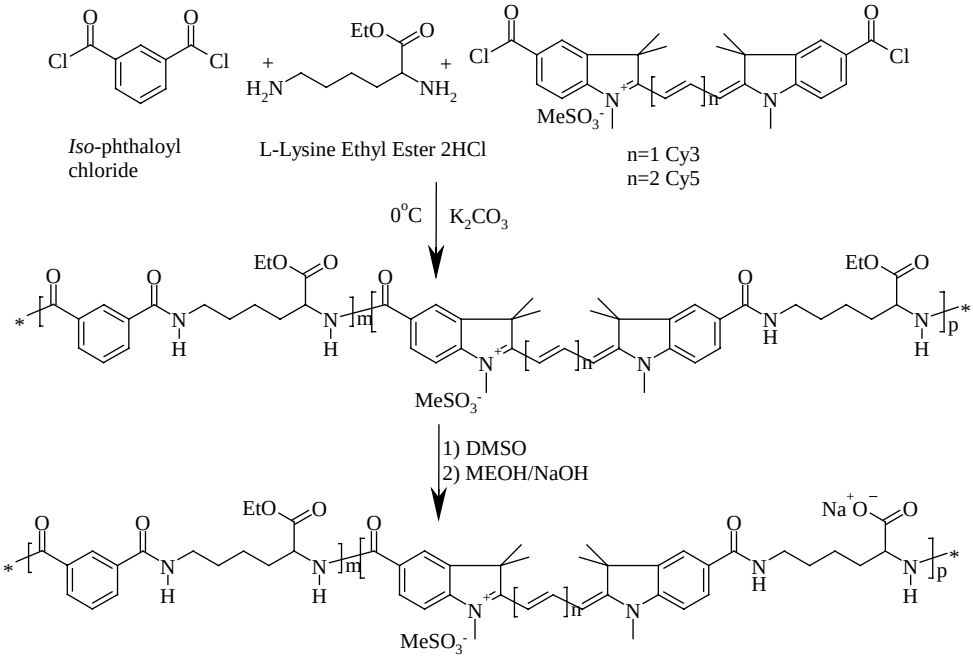
The yields of the purified cyanine fluorophores were low in both cases (~30%) reflecting the deactivating effect of electrophilic substituents on the heterocyclic indolenium salt [33]. The purified samples were analysed by electrospray mass spectrometry (positive ion mode) giving the expected parent

ion peaks at 445 Da for Cy3 ($C_{27}H_{29}N_2O_4$) and at 471 Da for Cy5 ($C_{29}H_{31}N_2O_4$). In both cases a smaller peak at +14 to the parent peak was seen (25% of main peak for Cy5 and 40% of main peak for Cy3). This is most likely due to partial esterification of the carboxyl functions by methanol catalysed with the sulphuric acid generated from the dimethyl sulphate. The dye materials produced according to the method above were used directly for polymer preparation and not purified further. From the ^{1}H NMR spectra of the crude Cy3 and Cy5 systems the N–CH_3 protons were clearly present at $\delta = 3.65$ and the 3 and 3′ methyl protons at $\delta = 1.7$–1.8. The first and second methane protons were centred at $\delta = 6.45$ and 8.58 for Cy 3 and at $\delta = 6.35$ and 8.65 for Cy 5. Additional assignments and integration, particularly of the aromatic peaks in the range $\delta =$ 7–8 were complicated by the multi-component nature of the crude dye stocks. FTIR analysis showed strong absorptions at 1704 cm^{-1} due to the carboxyl functions. Aromatic absorptions at 1610 and 820 cm^{-1} were also clearly visible.

2.2.3. Polydye synthesis

The polydyes were prepared using a modified method of Eccleston et al. [21,25]. The required amount of bis-carboxy acid derivatives of Cy3 or Cy3 and Cy5 in the case of the dual fluorophore containing polymers were suspended in chloroform and converted to their diacid chloride derivatives by reaction with excess oxaloyl chloride in the presence of one drop of DMF. Once the initial vigorous efflux of gas subsided, the suspension was heated gently using a hot air blower being careful to avoid reflux of the solvent. In the case of the Cy3 derivative the suspended solid appeared to completely dissolve whereas in the case of the Cy5 derivative the reaction appeared to proceed heterogeneously. The activated free dyes were diluted with cooled chloroform and the required amount of *iso*-phthaloyl chloride added. This organic phase was then reacted interfacially with an aqueous phase containing L-lysine ethyl ester and an acid acceptor. In a typical procedure, 0.54 g bis-carboxy Cy3 (1 mmol) were dispersed in 10 mL Chloroform and 0.25 mL oxaloyl chloride added (50% excess) together with one drop of DMF. After the initial gas evolution had ceased the suspension was warmed and a further 0.25 mL of oxaloyl chloride was added together with 10 mL acetone to achieve complete dissolution of the fluorophore. After stirring for 10 min 3.85 g *iso*-phthaloyl chloride (19 mmol) added to the activated fluorophore and allowed to dissolve. Separately 4.94 g of L-lysine ethyl ester, 2HCl (20 mmol) and 22 g of potassium carbonate (160 mmol) were dissolved in 100 mL deionised water and cooled until the appearance of ice crystals and then placed in a 1 L Waring laboratory blender vessel previously cooled in a −20 °C chest freezer. The blender was turned on at maximum speed and the acid chloride solution added. A red precipitate (purple in the case of Cy5) began to form from the rapidly stirred system almost immediately. The reaction was allowed to proceed for 30 min during which time the majority of the chloroform had evaporated. The coloured sticky precipitate was recovered by scraping with a spatula and washed with deionised water then dried over night in a vacuum oven at 50 °C. The ester groups were removed by methanolic sodium hydroxide treatment of the polymer in DMSO solution as reported previously [20].

The structures of the free dyes and polydyes are shown in Scheme 1. The loading yield of the dyes were determined by comparing the absorbance of poly-Cy3, poly-Cy5 and



n=1 1,1',3,3,3',3'-hexamethyl-5,5'dicarboxyindocarbocyanine methylsulphate (Bis Carboxy Cy3)
n=2 1,1',3,3,3',3'-hexamethyl-5,5'dicarboxyindodicarbocyanine methylsulphate (Bis Carboxy Cy5)

Scheme 1. Synthetic pathway for preparation of poly(L-lysine *iso*-phthalamide) with cyanine dye labels.

poly-Cy3,5 in deionised water to standard curves of Cy3 and Cy5 in deionised water at 557 and 656 nm, respectively.

2.3. Sample preparation

Poly(L-lysine *co*-bis-carboxy-Cy3 *iso*-phthalamide) (poly-Cy3) (6.96 wt% Cy3), poly(L-lysine *co*-bis-carboxy-Cy5 *iso*-phthalamide) (poly-Cy5) (1.43 wt% Cy5), and poly(L-lysine *co*-bis-carboxy-Cy3 *iso*-phthalamide-*co* bis carboxy-Cy5) (poly-Cy3,5) (7.12 wt% Cy3, 2.09 wt% Cy5) were purified by dialysis in deionised water for 2 weeks and then freeze–dried. Aqueous phase gel permeation chromatography (GPC) was conducted on a Viscotek system consisting of 2×30 cm ViscoGEL GMPW columns equipped with a VE3580 refractive index detector using 0.1 N $NaNO_3$ with 15% methanol as eluent and a flow rate of 1 mL min^{-1}. The system was calibrated with PEO standards giving apparent molecular weights (M_w) of 7600, 5800 and 7400 Da for poly-Cy3, poly-Cy5 and poly-Cy3,5, respectively. The low apparent molecular weights of the polydyes compared to the parent polymer (typical M_w 24,000 Da [23]) may, in part, be due to increased interaction of the Cy-doped polymers with the column material giving artificially high retention times. Cyanine derivatives are notoriously difficult to purify by chromatography due strong non-specific interaction with column materials [33]. Incomplete conversion of the diacid derivatives to the diacyl chlorides and the presence of mono ester Cy derivatives would also reduce the molecular weight relative to the parent polymer although it is expected that exhaustive dialysis would remove such low molecular weight material. For spectrophotometric analyses 0.1 M Na_2HPO_3 aqueous solution was mixed with 0.1 M NaH_2PO_3 aqueous solution to obtain 0.1 M phosphate buffer solutions (PBS) at pH varying from 4.0 to 7.0. All samples were filtered through a 0.45 μm filter prior to analysis.

Stock solutions (20 mg mL^{-1}) of the free dye and polydye solutions were prepared and diluted to the required concentrations with 0.1 M BPS at the specified pH for measurements.

2.4. Quantum yield measurements

The quantum yields of poly-Cy3 and the Cy3 monomer were determined by comparison of the wavelength-integrated intensities of the respective solutions with that of a reference rhodamine B (RhB) in deionised water [34]. The optical densities were kept below 0.05 to avoid inner filter effects. The concentrations of the RhB and Cy3 aqueous solutions were adjusted to match the optical density of a poly-Cy3 aqueous solution at a concentration of 0.01 mg mL^{-1} at a wavelength of 530 nm and the fluorescence emission spectra were recorded (λ_{ex} 530 nm) on a SPEX FluoroMax-3 spectrofluorometer (HORIBA JOBIN YVON, UK). The quantum yields of poly-Cy3 and the Cy3 monomer were then calculated using Eq. (1)

$$\Phi = \Phi_R \frac{I}{I_R} \frac{OD_R}{OD} \frac{n^2}{n_R^2} \quad (1)$$

where Φ represents the quantum yield, I is the integrated intensity, OD is the optical density, and n is the refractive index. The subscript R refers to the RhB reference fluorophore with known quantum yield of 0.48 [35].

2.5. Dynamic light scattering (DLS)

Dynamic light scattering experiments were carried out using a PD2000 dynamic light scattering instrument (Precision Detectors, USA) to determine the hydrodynamic diameter of poly-Cy3 aggregates in solution. The DLS instrument was equipped with an 800 nm laser light source, a 256 channel digital correlator and a sample chamber for a 1.0 mL quartz cuvette.

2.6. Absorption and steady-state fluorescence spectra

UV–vis absorption spectra were recorded on a Unicam UV1 spectrometer (Spectronic Unicam, UK). Steady-state fluorescence emission spectra of poly-Cy3 in PBS solution were obtained using a SPEX FluoroMax-3 spectrofluorometer (Horiba Jobin Yvon, UK) with slit widths of 1 nm for emission and 5 nm for excitation (λ_{ex} 565 nm, 10 mm×10 mm cuvette).

2.7. Fluorescence lifetimes

Fluorescence lifetimes of Cy3 and poly-Cy3 were determined using a frequency domain fluorescence lifetime imaging microscopy system (LIFA, Lambert Instruments, Netherlands) consisting of an Olympus IX50 inverted microscope equipped with a 520 nm LED excitation source and an intensified CCD detector (Lambert Instruments). Both the LED and the intensifier were modulated at a frequency of 40 MHz. A 1.0 μm Rhodamine 6G aqueous solution with a known lifetime of 4.11 ns was used as a reference [36]. Approximately, 0.1 mL of each sample solution was put on a microscope slide and illuminated near the centre of the drop to obtain fluorescence lifetime information. The fluorescence lifetime leads to a phase shift (ϕ) of the fluorescence waveform and a demodulation (m) of the emission light with respect to the excitation waveform. For every pixel, the fluorescence intensity was determined as a function of ϕ by recording a sequence of 12 images over a full modulation cycle in a scheme analogous to homodyne detection. A sine wave function was fitted through the sequence of recorded intensities in each pixel to determine ϕ and m from which lifetimes were determined using Eqs. (2) and (3)

$$\tau_\phi = \frac{1}{\omega} \tan \phi \quad (2)$$

$$\tau_m = \frac{1}{\omega} \sqrt{\frac{1}{m^2} - 1} \quad (3)$$

where τ_ϕ is the phase lifetime, τ_m the modulation lifetime, and ω the modulation frequency (in Hz) [37].

2.8. Non-radiative energy transfer (NRET)

NRET studies of poly-Cy3,5 and mixed poly-Cy3 and poly-Cy5 solutions were performed on a SPEX FluoroMax-3 spectrofluorometer. The concentration of poly-Cy3 and poly-Cy5 were adjusted to be equivalent to the Cy3 and Cy5 components of the poly-Cy3,5 solutions by matching the absorbance at 557 nm (λ_{ex} of Cy3) and 656 nm (λ_{ex} of Cy5), respectively. Fluorescence emission spectra were recorded at λ_{ex} 510 nm to minimise direct excitation of the Cy5 fluorophore. The ratio of emission maxima for Cy5 to Cy3 (I_{Cy5}/I_{Cy3}) was determined as a function of pH and polymer concentration.

3. Results and discussion

3.1. Quantum yield measurement

The emission spectra of aqueous solutions of poly-Cy3, Cy3 and the reference RhB with equivalent optical densities at λ_{ex} 530 nm are shown in Fig. 1. The integrated intensities were obtained from the emission spectra, and the quantum yields determined using Eq. (1). The quantum yields of poly-Cy3 and Cy3 were determined to be 0.53 and 0.08, respectively, using a value of 0.48 for the reference RhB. This seven-fold increase in quantum yield demonstrates that polymerisation results in enhanced fluorescence emission. This is in agreement with previous observations in a related system containing polymerised bis-amino Cy3 derivatives and is believed to result from a decreased mobility of the conjugated cyanine system upon inclusion within the backbone of a rigid polymer [21].

3.2. pH-Responsive fluorescence of polydye in solution

3.2.1. Steady-state fluorescence spectroscopy

Steady-state fluorescence measurements were performed to investigate changes in fluorescence emission intensities of poly-Cy3 with pH. The absorption and emission spectra of an aqueous solution of poly-Cy3 (0.05 mg mL^{-1}) at pH 6.0 are shown in Fig. 2. The variation of fluorescence emission intensity, integrated over the range of 400–850 nm, with polydye concentration and pH is shown in Fig. 3. For each concentration, there was an initial gradual increase in intensity with acidification down to pH 5, followed by a sharp decrease with further acidification to pH 4.0. This trend is consistent with previous observations on related fluorophore containing polymer systems and is ascribed to an initial enhancement of fluorescence output due to an increased micro-viscosity and

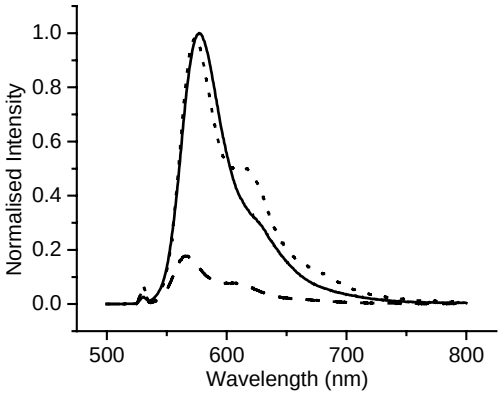


Fig. 1. Emission spectra of poly-Cy3 (…) and Cy3 (– – –) relative to RhB (—) in deionised water. The concentrations of the reference RhB and Cy3 aqueous solutions were adjusted to match the optical density of a poly-Cy3 aqueous solution at a concentration of 0.01 mg mL^{-1} at a wavelength of 530 nm and the resulting spectra were normalised to the RhB emission maxima.

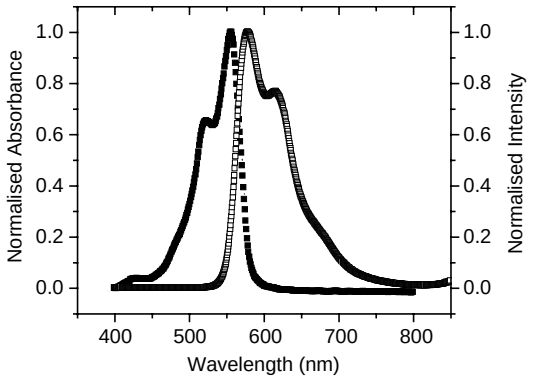


Fig. 2. Normalised absorbance (■) and emission (□) spectra of poly-Cy3 at a concentration of 0.5 mg mL^{-1} in 0.1 M PBS at pH 6.0.

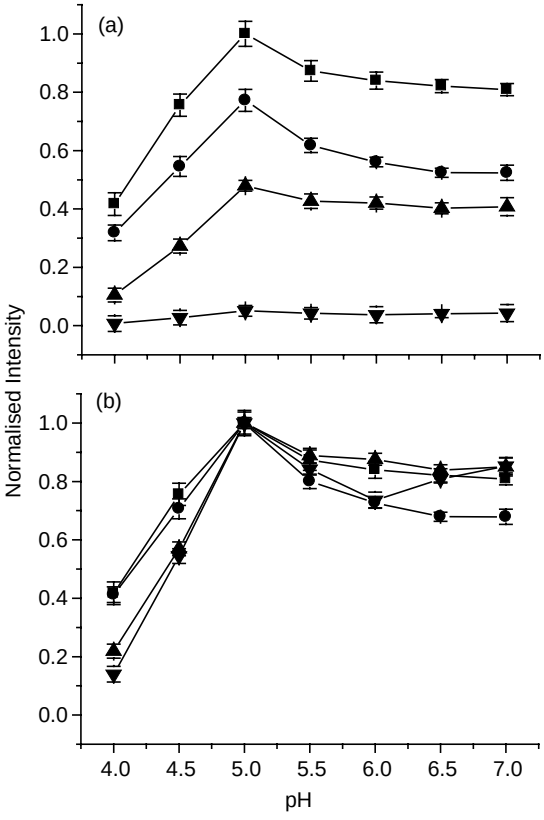


Fig. 3. Variation in emission intensity (integrated over 400–850 nm) of poly-Cy3 at concentrations of 1.0 (■), 0.5 (●), 0.05 (▲), and 0.005 (▼) mg mL^{-1} in 0.1 M PBS at pH from 4.0 to 7.0. Normalised with respect to poly-Cy3 solution with concentration of 1.0 mg mL^{-1} at pH 5.0 in (a) and normalised individually (b).

the development of hydrophobic domains offering protection from external quenchers followed by increased self quenching as fluorophores are brought into close proximity within increasingly compact structures as the pH is lowered further [21].

Further insight into the self quenching effect may allow improved design of molecular probes based on environmentally responsive polymeric systems. A plot of wavelength at fluorescence emission maximum (λ_{max}^{em}) as a function of pH is shown in Fig. 4(a). Interestingly, the emission spectrum was red shifted with increasing polymer concentration and displayed a further marked increase below a critical pH. At the lower concentrations studied (0.05 and 0.005 mg mL^{-1}), λ_{max}^{em} remained approximately constant (574 and 572 nm, respectively) in the range $4.5 < pH < 7.0$ but showed a further redshift (7 and 9 nm, respectively) to 581 nm as the pH decreased to 4.0. At higher concentrations the poly-Cy3 emission maxima were increasingly red shifted over the entire pH range investigated. In the range of pH from 7.0 to 5.0 there was a gradual redshift of 1.5 nm from 578.5 to 580 nm at a concentration of 0.5 mg mL^{-1} and similarly from 582.5 to 584 nm at a concentration 1 mg mL^{-1}. Between pH 5.0 and 4.0 a steeper increase was observed from 580 to 587.5 nm (0.5 mg mL^{-1}) and 585 to 589 nm (1.0 mg mL^{-1}).

The redshift indicates a reduction in the energy of the excited fluorophore prior to emission which could be a result of sequential reabsorption and loss of energy and/or energy reduction due to π–π stacking between conjugated Cy3 segments [38,39]. The probability of π–π stacking between Cy3 groups on a single polymer chain in the expanded state, i.e. above pH 4.5, is low as is the likelihood of reabsorption at low concentration. As the concentration increases the likelihood of reabsorption increases resulting in an increasingly redshifted emission spectrum. Below the critical pH for the onset of hypercoiling, Cy3 groups on single polymer chains can be brought into close proximity even at low concentration allowing potential π–π stacking and non-radiative energy transfer as well as increasing the likelihood of reabsorption (radiative energy transfer) between non-stacked fluorophores. At higher fluorophore concentration, i.e. increased polymer concentration or upon polymer collapse, the amount of reabsorption would increase and enhance energy loss. However, the relative decrease in fluorescent output and, the degree of red shift on polymer collapse are both lower at higher polymer concentration (Figs. 3(b) and 4(b)). This could be explained by a reduction in the contribution of π–π stacking of the fluorophores due to steric effects in multipolymer aggregates formed as the concentration increases. Indeed the formation of polymer aggregates at the higher polymer concentrations, at which these effects were observed, is strongly indicated by lifetime and FRET measurements, subject of Sections 3.2.2 and 3.4, respectively.

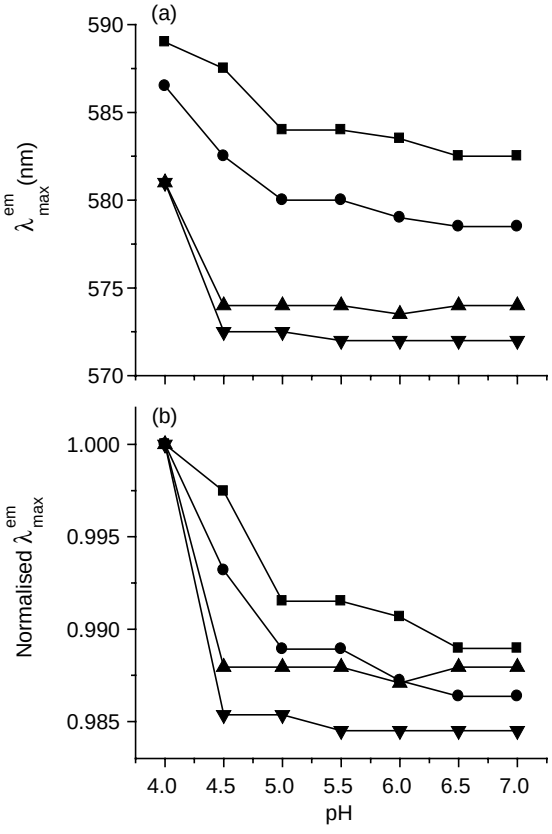


Fig. 4. Variation in wavelength at which maximum fluorescence emission occurs (a) and normalised λ_{max}^{em} (b) of poly-Cy3 with concentrations of 1.0 (■), 0.5 (●), 0.05 (▲), and 0.005 (▼) mg mL^{-1} in 0.1 M PBS at pH from 4.0 to 7.0.

3.2.2. Fluorescence lifetimes

The fluorescence lifetime is the average time that a molecule remains in an 'excited state' upon absorption of light before returning to the ground state. Whilst steady-state intensity measurements may be affected by factors such as scattering and self-absorption [37] the fluorescence lifetime is indicative of changes in the immediate environment of the fluorophore and is a useful tool for probing conformational change and inter-molecular association of responsive polymer systems.

The fluorescence lifetime of the Cy3 monomer in aqueous solution is independent of pH ($4.0 \leq pH \leq 7.0$) and

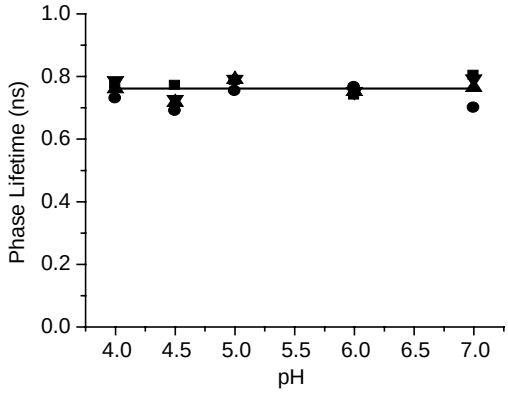


Fig. 5. Fluorescence phase lifetime τ_ϕ for Cy3 free dye solutions with concentration of 0.05 (■), 0.025 (●), 0.005 (▲) and 0.0025 (▼) μm in 0.1 M PBS as a function of pH.

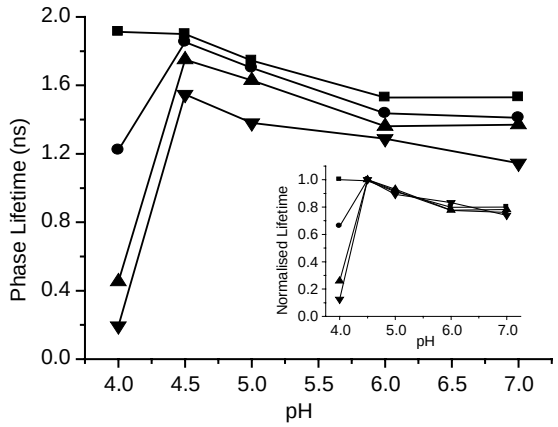


Fig. 6. Variation in fluorescence phase lifetime τ_ϕ and normalised τ_ϕ (inset) of poly-Cy3 solutions at concentrations of 2.0 (■), 1.0 (●), 0.5 (▲), and 0.05 (▼) mg mL^{-1} in 0.1 M PBS as function of pH.

concentration (0.0025–0.05 μm) remaining effectively constant at 0.75 ns (Fig. 5). Thus, since the Cy3 monomer is insensitive to pH and concentration effects within the ranges studied variations in the fluorescence lifetime of poly-Cy3 must be due to the influence of conformational changes of the polymer.

The variation of τ_ϕ as a function of pH for four concentrations of poly-Cy3 solutions is shown in Fig. 6. The fluorescence lifetime of poly-Cy3 was dependent on both pH and concentration, particularly below pH 4.5. Solutions with higher polymer concentrations had longer fluorescence lifetimes, indicative of a reduction in the number of quenching events. As the pH was decreased the fluorescence lifetime gradually increased between pH 7.0 and 4.5 followed by a sharp decrease below pH 4.5 with the notable exception of the 2.0 mg mL^{-1} sample where the lifetime continued to increase below pH 4.5. Interestingly, if one examines the relative changes in the normalised fluorescent lifetime with pH at the four concentrations studied (Fig. 6 inset) it can be seen that the magnitude of reduction in lifetime reduces as the concentration increases and eventually disappears at 2.0 mg mL^{-1}. As the solution becomes increasingly dilute then the likelihood of inter-molecular interactions is reduced thus these observations can be rationalised by assuming that intra-molecular association of fluorophores upon polymer collapse results in increased self quenching and thus a reduction in the fluorescence lifetime and the steady state fluorescence of the fluorophores. As the concentration of the poly-Cy3 increases then the likelihood of inter-molecular interactions increases and the formation of multipolymer aggregates takes place. Enhanced association and hydrophobic domain formation of the polymer backbone within these multipolymer aggregates may reduce the degree of fluorophore overlap possible in single molecular hypercoils and offer protection from external quenchers. In addition the mobility of the fluorophores in the larger multimolecular assemblies may be reduced due to 'crystallisation' of the hydrophobic domains as noted previously for multimolecular micellular aggregates formed from PEGylated poly(L-lysine *iso*-phthalamide) [23]. Thus, at low concentrations the pH dependence of the fluorescent behaviour is dominated by intra-molecular quenching events whereas at higher concentrations inter-molecular aggregation eventually reduces the ability of the fluorophores to align. This trend mirrored the variation in steady-state fluorescence intensity with pH (Fig. 3) for the three lower concentrations and is further support of the occurrence of a hypercoiling conformational transition between pH 5.0 and 4.0. At the higher concentration of 2.0 mg mL^{-1} the fluorescent lifetime of the fluorophores continues to lengthen within the aggregates formed but the increased likelihood of reabsorption, the so called inner filter effect, would explain the overall reduction in steady state fluorescence discussed in the previous section.

3.3. Dynamic light scattering

It has been shown previously that poly(L-lysine *iso*-phthalamide) can adopt a hydrophobically stabilised tightly coiled structure upon partial neutralisation of the pendant carboxyl groups prior to aggregation and precipitation [15,20]. Dynamic light scattering (DLS) was employed to determine if aggregation was taking place at elevated polymer concentrations and the effect of pH on polymer conformation and aggregation. DLS of poly-Cy3 (5.0 mg mL^{-1}) at pH 6.0 revealed two distinct particle size distributions at 100 and 267 nm. Upon acidification to pH 4.0 there was a marked reduction in the average size of the distributions to 62 and 185 nm coupled with a considerable broadening of the larger

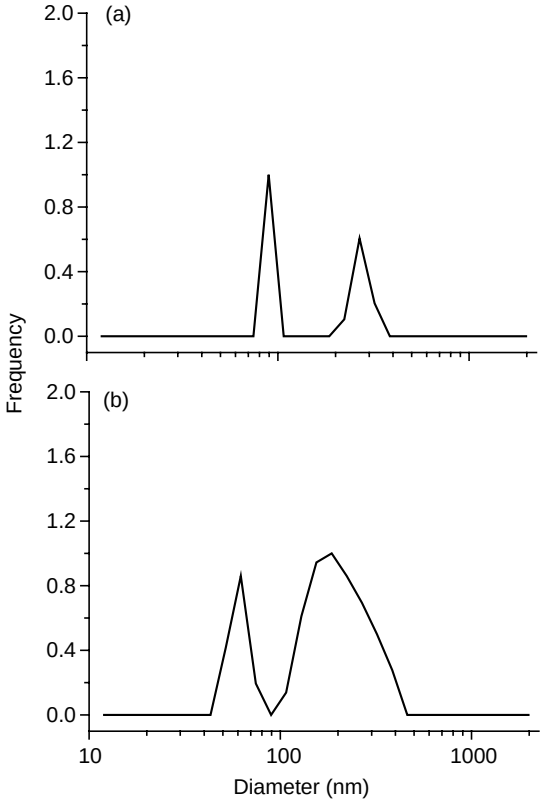


Fig. 7. Hydrodynamic diameter of poly-Cy3 at a concentration of 5.0 mg mL^{-1} at pH 7.0 (a) and pH 4.0 (b).

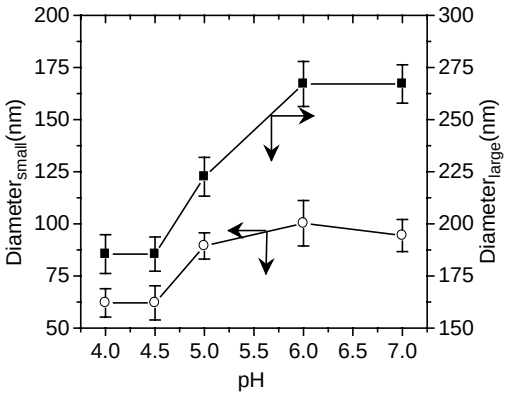


Fig. 8. Variation of mean hydrodynamic diameter of poly-Cy3 aggregates formed at a concentration of 5.0 mg mL^{-1} in PBS as a function of pH.

particle size distribution (Fig. 7). The variation of the mean size of each population over the pH range 4.0–7.0 is shown in Fig. 8. A significant reduction in the size of both populations was noted as the pH decreased from 6.0 to 4.5, i.e. within the range of pK_as previously noted for the poly(L-lysine *iso*-phthalamide) system. These observations are consistent with Cao et al. [40] who noted a bimodal particle size distribution for sodium alginate above a concentration of 2.5 mg mL^{-1}. They proposed that neutralisation of the carboxylate groups of the polysaccharide reduced the stabilisation of smaller polymeric assemblies, which subsequently aggregated into larger assemblies. The relatively high hydrophobicity of the poly(L-lysine *iso*-phthalamide) system of the present study would account for the increased sensitivity of the assembly size to pH. The bimodal particle size distribution was confirmed by AFM analysis of the samples (data not shown).

3.4. Non-radiative energy transfer (NRET)

The process of non-radiative energy transfer from a donor fluorophore to an acceptor chromophore over short distances (10–90 Å) is a useful tool to investigate the conformational transitions of fluorescently labelled polymers [31,32,40]. If the acceptor is fluorescent the degree of NRET can be measured from the fluorescence emission of the acceptor, i.e. via fluorescence resonant energy transfer (FRET). Thus, the degree of energy transfer from a Cy3 donor to Cy5 acceptor was used to probe the pH mediated intra- and inter-molecular association of dual fluorophore containing poly-Cy3,5 and mixtures of poly-Cy3 and poly-Cy5 at various pH values and concentrations. In the dual fluorophore polymer system FRET can occur both due to intra-molecular association and inter-molecular aggregation whereas in the single fluorophore polymer mixture FRET can only take place upon aggregation. Thus, these measurements provide potential for a further differentiation between intra- and inter-molecular effects.

The ratio of Cy5 emission to Cy3 emission (I_{Cy5}/I_{Cy3}) represents the energy transfer efficiency between donor and acceptor molecules. An excitation wavelength of 510 nm was employed to minimise direct excitation of the acceptor the absorption maximum of which occurred near 650 nm. The

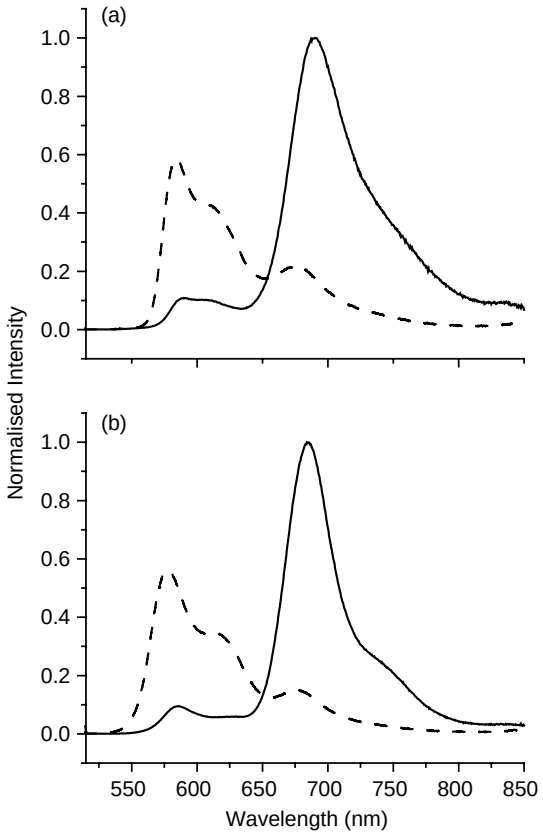


Fig. 9. Fluorescence emission spectra of dual fluorophore containing poly-Cy3,5 solution (a), and a mixed solution of poly-Cy3 and poly-Cy5 (b) at concentrations of 0.5 mg mL^{-1} for pH 4.0 (—) and pH 7.0 (– – –).The emission intensities for both systems were normalised to the maximum fluorescence intensity at pH 4.0 (λ_{ex} 510 nm).

normalised fluorescence emission spectra of poly-Cy3,5 and mixed poly-Cy3/poly-Cy5 solutions (total polymer concentration of 0.5 mg mL^{-1}) at pH 4.0 and 7.0 are shown in Fig. 9. λ^{em}_{max} was ~700 nm for Cy5 and ~580 nm for Cy3. In both the mixed single fluorophore and the dual fluorophore systems the emission intensity of the Cy5 acceptor was more intense at pH 4.0 then at pH 7.0, whilst the Cy3 donor signal was reduced, indicating that efficient energy transfer was taking place.

The variation of (I_{Cy5}/I_{Cy3}) with pH at three concentrations is shown in Fig. 10. In both dual and mixed polymer systems, the ratio of intensities is close to zero between pH 5.0 and 7.0 indicating insignificant energy transfer between donor and acceptor fluorophores. After a minor increase from pH 5.0 to 4.5, the energy transfer efficiencies increase dramatically towards pH 4.0.

Clearly in the mixed single fluorophore system FRET is only possible via inter-molecular interactions between donor and the acceptor whereas in the dual fluorophore system FRET can occur both on an intra-molecular level as well as following aggregation. We are currently in the process of exploiting such polymer systems which 'switch on' in response to environmental triggers for biodiagnostic applications. An advantage of the current system over others reported in the literature is that it is reversible and thus capable for monitoring dynamic biophysical processes. In contrast to probes which are

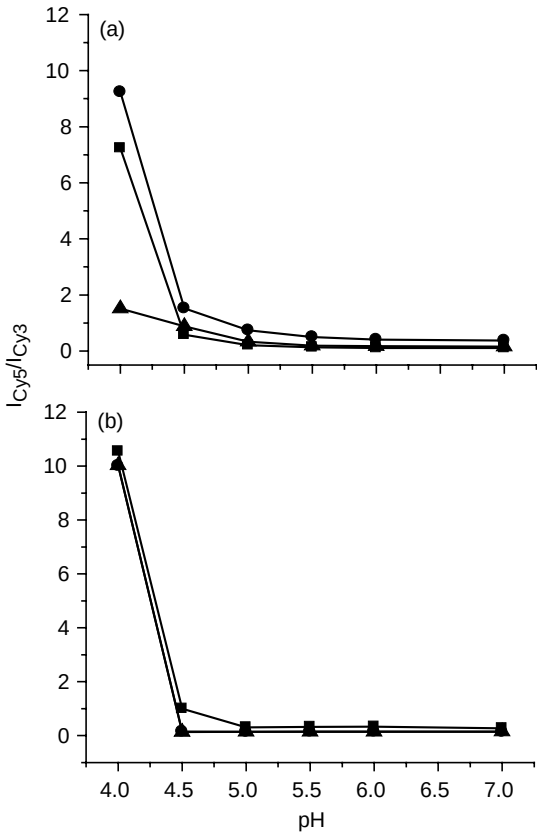


Fig. 10. Ratio of maximum fluorescence intensity of Cy3 donor and Cy5 acceptor (I_{Cy5}/I_{Cy3}) as a function of pH for dual fluorophore containing poly-Cy3,5 solution (a), and mixture solution of poly-Cy3/poly-Cy5 (b) at concentrations of 0.005 (■), 0.05 (●), and 0.5 (▲) mg mL^{-1} in 0.1 M PBS (λ^{ex} 510 nm).

'intrinsically on' and switch off on an external trigger, probes that use emission from sensitised acceptor molecules offer positive signal on very little background thus dramatically improving contrast and sensitivity. These properties can be further enhanced by appropriate selection of donor to acceptor loading ratios on the diagnostic probe. For example, FRET signals can be maximised by designing probes, which efficiently suppress residual donor background fluorescence via increased degrees of donor selfquenching in the collapsed state. Concomitantly, the acceptor can be protected from external quenchers. Furthermore, For each system there is an optimal ratio of donor to acceptor loading on the probe, which maximises FRET and minimises trapping of acceptor signal. Further possibilities may be realised through techniques employing lifetime measurements, which is particularly attractive due to increasing availability of fast modulation light emitting diode technology [41].

4. Conclusions

The effect of pH and concentration of poly(L-lysine *iso*-phthalamide) containing bis-carboxy cyanine fluorophores on steady-state fluorescence intensity, fluorescence lifetime and non-radiative energy transfer (NRET) efficiency were studied to obtain a detailed picture of polymer conformational dynamics allowing us to study the intra-molecular association and inter-molecular self-assembly of the pH-responsive pseudopeptide in aqueous solution. The single fluorophore containing system showed a gradual increase then a rapid decrease in steady state fluorescence coupled with a sharp red shift in the emission maxima below a critical pH over the whole concentration range studied. This suggests an initial reduction followed by a rapid increase in the amount of fluorophore quenching due to both non-radiative and radiative processes (inner filter effects). Conversely whilst a reduction in the fluorescent lifetime of the cyanine fluorophores occurred below the critical pH at relatively low concentration this effect diminished and eventually disappeared with increasing concentration indicating a reduction in the amount of non-radiative energy transfer with concentration. It was noted that the emission maxima wavelength showed a positive correlation with concentration both prior to and below the critical pH. Dynamic light scattering indicated a tendency for multi-molecular polymer assemblies with a bimodal size distribution to form at elevated concentration and the size of the assemblies was shown to be dependent on pH. We conclude from this data that below the critical pH poly(L-lysine *iso*-phthalamide) collapses to a hydrophobically stabilised system and that at low concentration this association is predominantly intra-molecular allowing a high degree of aggregation of the co-polymerised fluorophores. As the concentration increases, inter-molecular association of the polymer backbone increases and the degree of intra-molecular collapse decreases thus sterically impeding aggregation of the fluorophores. Thus, the reduction in steady state fluorescence is ascribed to a combination of non-radiative energy transfer between fluorophores at low concentrations with increasing contributions from radiative energy transfer as the concentration increases.

These conclusions are supported by the efficiency of NRET from Cy3 to Cy5 in a dual fluorophore and a mixture of single fluorophore containing polymers. Above the critical pH for onset of polymer collapse electrostatic repulsion along the polymer backbone and between polymer chains prevents either intra-molecular or inter-molecular fluorophore overlap. In contrast, the polymers displayed very high tendency for NRET under acidic conditions in both intra-polymer hydrophobic domains and inter-polymer aggregates.

Future work will focus on the investigation of cellular uptake of the polydyes for in vivo diagnostics and drug delivery applications where the enhancement in quantum yield could be harnessed in ultrabright probes for in vivo detection of diseased tissue by optical means.

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