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Optical characteristics of responsive biopolymers; co-polycondensation of tri-functional amino acids and Cy-3 bis-amine with diacylchlorides

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Abstract

The synthesis and fluorescence behaviour of a range of amphiphilic poly(L-lysine co-bis-amine-Cy-3 *iso*-phthalamide) polymers that incorporate low levels of Cy-3 dye co-monomer (0.5–2.1% (w/w)) in their backbone have been investigated in aqueous solution over a range of pH values. The desired functionality of these biocompatible, hydrophobically modified polyelectrolytes 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. Thus, the polymer changes from an extended conformation at high degrees of ionisation to a compact conformation stabilised by hydrophobic association at low degrees of ionisation and eventually precipitates from solution. At intermediate degrees of ionisation, such polymers exhibit amphiphilic properties. A bis-functional cyanine fluorophore derivative, co-polymerised within the polymer backbone, is demonstrated to act as a fluorescent reporter on the conformational state of the polymer. The materials have higher intrinsic fluorescence intensity per fluorophore monomer over a broad range of concentrations than bis-amine Cy-3 and their maximum fluorescence intensity is up to eight fold higher than the maximum intensity of bis-amine Cy-3, which is limited by quenching. It is also shown that the free fluorophore can be used to probe the conformation of unlabelled poly(L-lysine *iso*-phthalamide) through electrostatic interaction with the polymer. The technique allows rapid spectrophotometric determination of polymer conformation and offers the potential of an environmentally sensitive molecular pH probe for in vivo use.

Keywords: Biocompatible polyamides; Amino acids; Fluorescence

1. Introduction

The potential of water-soluble polymers to mediate drug delivery is well appreciated [1], but their use in medical imaging is relatively novel. We describe a class of biocompatible, environment-responsive co-polymers containing Cy-3-dye moieties that display a conformation dependent fluorescence and which might be exploited for imaging.

Amphiphilic macromolecules containing carboxyl groups together with pendant hydrophobic groups can change conformation in response to pH [2,3]. Thus, when the pH is sufficiently high electrostatic repulsion between carboxylate anions overcomes the association of the hydrophobic groups and results in an extended conformation. At lower pH loss of charge allows intra-molecular hydrophobic association and the polymer chain collapses to a compact conformation. In this way, a conformation dependent functional property can be made to switch on or off in response to small changes in the environment.

Metabolite-derived biocompatible polymers have been reported [4–7] and their use for drug targeting has been described [8,9]. We have synthesised such materials that additionally possess amphiphilic properties [10]. Amino acids containing both α- and ω-amine groups were co-polymerised with a range of hydrophobic dicarboxylic acids. The resulting polymers comprised an amide backbone with pendant hydrophobic and hydrophilic groups and displayed a pH dependent conformation. By variation of the hydrophobe and by partial esterification of the carboxyl functions, a range of polyamides were synthesised that changed molecular conformation in aqueous solution over the pH range 4.0–5.8. Furthermore, cytotoxicity tests with a

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range of mammalian cell lines (COS1, CHO, A2780, MAC-16 and C-26) confirmed that poly(L-lysine *iso*-phthalamide) co-polymers are well tolerated at physiological pH [11].

Delaire et al. [12] describe pendant fluorophore-modified, water-soluble co-polymers of methacrylic acid and vinyl-diphenylanthracene with pH dependent fluorescent properties. Pendant dye modified polyesters with pH dependent NRET fluorescent properties have also been described [13,14], and pH dependent fluorescence behaviour has been reported for cationic [15], solid phase [16] and amphiphilic polymers [17,18]. In these examples, the distance between fluorophores in the extended chain conformation reduces non-radiative energy transfer processes. When conformational change is triggered the fluorophores are brought into close proximity within the hydrophobic core of the molecule, and the change in their environment leads to a modulation of fluorescence.

To exploit this phenomenon within a biocompatible polymer scaffold, we have synthesised a range of fluorescent poly(L-lysine co-bis-amine-Cy-3 *iso*-phthalamide) co-polymers that contain Cy-3 bis-amine fluorophores within their backbone. In related work, we have imaged the entry of these polymers into CHO cells by confocal microscopy [11]. Such internalisation of macromolecules occurs by endocytosis [19], which is often upregulated in tumours [20], where polymers can accumulate [9,21–24]. By tailoring the pH response of the polymer to the low pH within endosomes [25,26], the change in optical signal might permit ex vivo imaging. In this paper, we describe studies on the conformation dependent fluorescence behaviour of such polymers.

2. Experimental section

Polymers were synthesised according to previously published methods [10]. Typically a solution of *iso*-pthaloyl chloride in acetone (0.2 M) was added to a rapidly stirred, aqueous solution of L-lysine (0.2 M, 0 °C) and potassium carbonate (0.6 M) that acts as an acid acceptor. After 30 min, the acetone was removed under vacuum. The crude polymer solutions were concentrated to a volume of 100 ml using a Millipore miniplate™ containing a cellulose diafiltration membrane with a molecular weight cut off of 3 kDa and then dialysed with deionised water to remove inorganic salts, low molecular weight oligomers and residual organic solvent. The purified polymers were lyophilised to give the potassium salt and stored at 4 °C.

Fluorophore labelled polymers ('Polydyes' or PD's hereafter) were prepared by co-polymerising L-lysine and a bis-amine Cy-3 cyanine fluorophore derivative with *iso*-phthaloyl chloride. The polymerisation technique was analogous to that for the naked polymer. By way of an example, the synthesis of PD20 proceeded as follows: 1.46 g L-lysine (10 mM), 0.21 g bis-amine Cy-3 (0.5 mM) and 4.29 g potassium carbonate (31 mM) were dissolved in 50 ml deionised water and cooled to 0 °C (note, the Polydye number denotes the molar ratio of L-lysine to bis-amine Cy-3 in the reaction mixture, here 20:1). 2.132 g *iso*-phthaloyl chloride (10.5 mM) was dissolved in 50 ml acetone, pre-cooled to 0 °C. The organic solution was added to the rapidly stirred aqueous phase (Waring blender, full speed) and stirring was maintained for 30 min. After this, the acetone was removed under vacuum and the aqueous phase was dialysed in an ultrafiltration unit (Millipore, MW cut off 3 kDa) with four volumes of deionised water to remove salts and oligomeric material. The resulting solution was then lyophilised to give a deeply purple solid. Polymers with higher molar ratios of L-lysine to bis-amine Cy-3 were prepared analogously.

Absorption spectroscopy was used to determine the fluorophore content of the Polydye samples. Beer–Lambert plots were determined for each of the Polydye samples at 553 nm using a Perkin–Elmer UV–Vis spectrophotometer. The fluorescence output of the labelled polymers was determined using either a Cytofluor™ fluorescent plate reader ($\lambda_{ex} = 530/25$ nm, $\lambda_{em} = 580/50$ nm) or an Aminco SPF-125 spectrofluorimeter ($\lambda_{ex} = 550$ nm, $\lambda_{em} = 575$ nm). The fluorimeter was equipped with a flow-cell for spectrophotometric titrations, and samples of the fluorescent probe or solutions of the base polymer in the presence of free fluorophore were titrated with 1.0 M HCl using a radiometer TIM 900 autotitrator equipped with a 5 ml burette. The Polydye solution from the titration vessel was circulated through the flow cell using a peristaltic pump.

Gel permeation chromatograms were obtained for each Polydye sample using 2 × 30 cm Viscotek GMPW column with elution at 30 °C by a 0.2 M sodium nitrate/0.01 M sodium dihydrogenphosphate buffer adjusted to pH 7.0 and refractive index detection. Molecular weights were determined relative to PEG/PEO standards. To further investigate molecular weights, gel permeation chromatograms for Polydye 40 were obtained under similar conditions using 0.1 M sodium nitrate/15% methanol as eluent and a triple detector system employing low angle light scattering, refractive index and viscometric detection.

For the gel filtration analyses, 1 l of 0.1 M phosphate buffered saline was run through a Superdex 75 column (Hiload 16/60, Pharmacia Biotech), followed by 1 ml of a 1 mg ml^{-1} solution of Polydye 20 in 0.1 M PBS. Samples were collected at regular time intervals and their fluorescence was measured (Cytofluor plate reader, ex ~530/25 nm, em ~590/20 nm). This procedure was repeated for free Cy-3 bis-amine and fluorescence measurements were taken in the same manner.

Poly acrylamide gel electrophoresis was undertaken using a Biorad Mini-PROTEAN II gel electrophoresis kit. Samples of each Polydye, (1 g l^{-1}), were mixed with an equal volume of loading buffer (40% sucrose) and loaded into the wells of a pre-cast poly acrylamide gel. Cy-3 bis-amine and a mixture of free Cy-3 bis-amine with poly(lysine

iso-phthalamide) were electrophoresed as controls. The gel was then developed at 200 V in a Tris/glycine buffer at pH 7.4. The gels were imaged using a LEADseeker™ (Nycomed–Amersham) high definition fluorescence CCD image acquisition unit ($\lambda_{ex} = 535$ nm, $\lambda_{em} = 595$, 10 nm band widths on excitation filter).

3. Results and discussion

3.1. Polydye characterisation

The synthesis and characterisation of the naked poly(L-lysine *iso*-phthalamide) co-polymer (Fig. 1) has been described previously [10]. FT-IR analysis of the naked polymer precipitates showed characteristic absorptions due to the carboxylic acid C=O stretch ($\sim 1710\ cm^{-1}$) with strong absorption at $\sim 1640\ cm^{-1}$ (amide band I) and at $\sim 1542\ cm^{-1}$ (amide band II). Weight average molecular weights (M_w) of the pre-dialysed samples, determined by gel permeation chromatography in dimethylformamide at 80 °C and calibrated with polyethylene glycol/polyethylene oxide standards, were in excess of 40 kDa (but see discussion below). The number average was much lower indicating that the samples were polydisperse and this was confirmed by the observation that M_w for the polymer in the diafiltrate was typically ~ 10 kDa.

Since, the rate of Cy-3 bis-amine monomer incorporation into the polymer might be different from that of lysine, the possibility exists that the distribution of fluorophores in the polymers might be non-random, with local incorporation of multiple fluorophores leading to fluorescence quenching even in the extended polymer chains. To reduce the probability of such effects, low levels of fluorophore incorporation were used such that the maximum mean level of dye incorporation in the fluorescent co-polymers reported here corresponded to just one bis-amine Cy-3 monomer per 20.6 kDa of polymer (PD20).

NMR analysis of the Polydye samples was attempted to determine the dye loading and distribution of fluorophores within the polymer backbone. However, spectral broadening due to sample viscosity, coupled with the low levels of dye incorporation, degraded resolution in the proton spectra such that the determination of fluorophore to polymer ratios and the relative distribution of the fluorophores was not possible.

Gel permeation chromatography of the Polydye samples showed that the distribution of column retention times was comparable for each of the polymers (Fig. 2(a)), though PD40 eluted slightly later. Relative to PEG/PEO standards, the molecular weights of the materials appeared low (<6000 Da) though the conformation and solvation of the standards at pH 7.0 is unrepresentative of the charged Polydyes (see Ref. [27] for discussion). To investigate this issue, GPC analysis of a sample of Polydye 40 ($6.14\ mg\ ml^{-1}$) was conducted using a combined light scattering, viscometric and refractive index detection system. The molecular weights calculated from the light

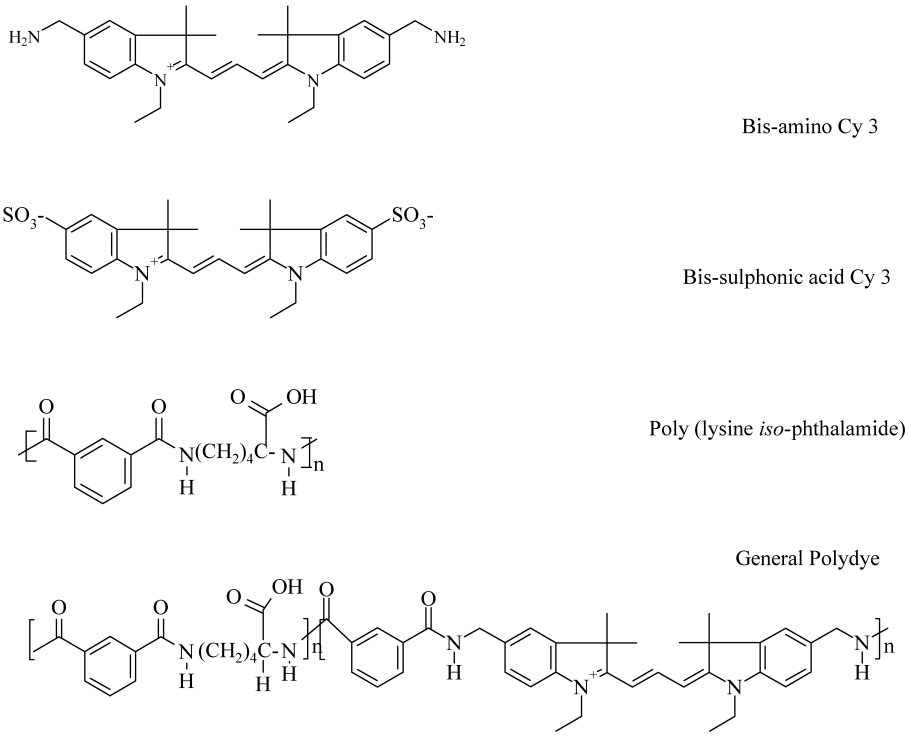

Fig. 1. Chemical structures of the derivatised Cy-3 monomer, bis-amino Cy-3, employed for the synthesis of Polydyes in which the fluorophore was polymerised within the backbone of a poly(lysine *iso*-phthalamide) polymer with a range of loadings. Also shown is the structure of the bis-sulphonic acid Cy-3 derivative.

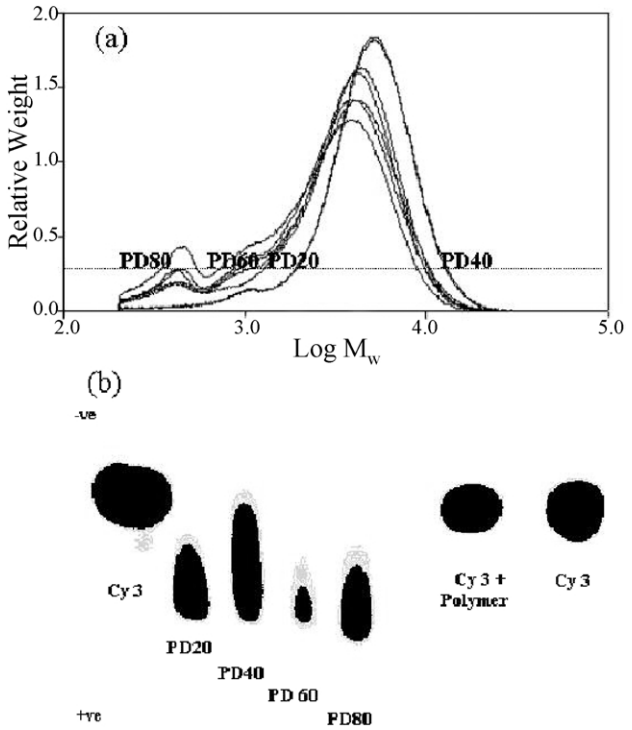

Fig. 2. (a) Relative molecular weight distributions for Polydyes 20, 40, 60 and 80 (PD20, PD40, PD60 and PD80) determined from aqueous gel permeation chromatograms using 2 × 30 cm Viscotek GMPW column with elution at 30 °C by a 0.2 M sodium nitrate/0.01 M sodium dihydrogenphosphate buffer adjusted to pH 7.0. (b) Fluorescence image of poly acrylamide gel electrophoresis of bis-amino Cy-3 monomer, a mixture of bis-amino Cy-3 monomer and poly(lysine-*iso*-phthalamide) and PD20, PD40, PD60 and PD80, indicating the relative electrophoretic mobilities of fluorophores in free solution (in the absence and presence of polymer) and of the fluorophore polymerised Polydyes.

scattering data are shown in Table 1, supporting much higher values in the region of 100 kDa. Increased chain stiffness in the Polydyes is indicated by a Mark-Houwink 'a' value of 1.15 (c.f. 0.7 for polystyrene in THF, a classic random coil) and this is anticipated from the electrostatic repulsion between charged carboxyl groups along the polymer backbone.

Two additional studies were conducted to confirm the conjugation of the fluorophore within the poly(L-lysine *iso*-phthalamide) backbone. First, the elution behaviour of PD20 and unconjugated Cy-3 bis-amine from a Superdex-75 gel permeation column were separately studied. It was observed that Cy-3 bis-amine eluted around fraction 50, whilst PD20 eluted in a broad band from fraction 15–45 with no peak evident at fraction 50 (data not shown). In a second study, SDS–PAGE gel electrophoresis of each of the Polydyes, a poly(lysine *iso*-phthalamide)/Cy-3 bis-amine mixture and unpolymerised Cy-3 bis-amine was conducted (Fig. 2(b)). All the Polydye samples displayed smeared bands, but a clear distinction was made between these samples that moved to the anode and the Cy-3 bis-amine controls that moved to the cathode both in the presence and absence of poly(lysine *iso*-phthalamide). No evidence of association of free Cy-3 bis-amine to poly (lysine *iso*-phthalamide) was observed in the mixed sample during electrophoresis. The electrophoresis bands for PD20, 60 and 80 were similar, as anticipated from the GPC data, and there was no evidence of residual non-polymerised Cy-3 bis-amine in these samples. For PD40, the longer tail indicates the presence of polymer molecules with a lower overall charge consistent with the relatively high fluorophore to polymer ratio (since each fluorophore contains a quaternary nitrogen atom).

Table 1
Mean molecular weight (M_w), number average molecular weight (M_n) and *z*-average molecular weights for two samples of Polydye 40 determined by GPC with light scattering, refractive index and viscometric detection. Also indicated are the sample polydispersities

	M_w (Da)	M_n (Da)	M_z (Da)	Polydispersity
Run 1	100,900	89,360	118,800	1.13
Run 2	103,000	90,260	123,000	1.14

3.2. Fluorescence output of polymer samples

We first sought to establish the interaction between naked polymer and Cy-3 bis-amine, and its influence upon fluorescence by monitoring the relative fluorescence intensity of a solution of the test poly(lysine *iso*-phthalamide) and the bis-amine Cy-3 derivative in PBS at pH 7.4. Under such conditions the cationic amino groups interact electrostatically with the anionic carboxyl groups. The relative fluorescence intensity of the bis-amine Cy-3 derivative was enhanced upon binding to the polymer, presumably due to the reduced mobility of the bound fluorophore [28]. Such fluorescence was strongly dependent on the Cy-3 bis-amine concentration and varied little above a critical polymer concentration (Fig. 3).

The excitation and emission spectra of the poly(L-lysine co-bis-amine-Cy-3 *iso*-phthalamide) probes were similar to that of the unpolymerised bis-amine Cy-3 derivative,

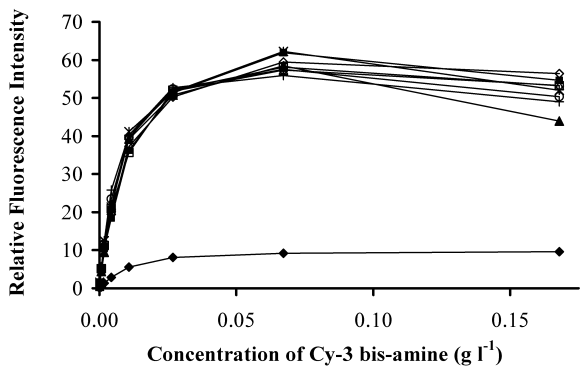

Fig. 3. Variation of relative fluorescence intensity of the Cy-3 bis-amine derivative measured at pH 7.4 in aqueous solution using a Cytofluor plate reader (λ_{ex} = 535 nm and λ_{em} = 570 nm) for a range of concentrations of poly(lysine *iso*-phthalamide); 0.0 (◆), 0.64 (▲), 0.8(+), 0.96 (○), 1.12 (*), 1.28 (■), 1.44 (□) and 1.6 (◇) g l^{-1}.

although the absorption and emission maxima were shifted slightly to higher wavelengths (λ_{ex} from 550 to 553 nm, λ_{em} from 570 to 574 nm), consistent with their reduced mobility within the polymer backbone (Fig. 4). The concentration dependence of the relative fluorescence intensities of each of the dialysed Polydye probes was determined in phosphate buffer at pH 7.0 (Fig. 5). Two approaches were adopted to compare the fluorescence output of the different Polydyes. First, the concentration of single fluorophore containing polymer segments was estimated from the relative concentrations of bis-amine monomers (L-lysine and Cy-3 bis-amine) in the reaction mixture. On this basis, there was little difference in fluorescence between PD20, 60, and 80, though PD40 showed a higher fluorescence output (Fig. 5(a)). However, in view of the marked difference in chemical structure and size of the L-lysine and bis-amine Cy-3 monomers the implicit assumption of stoichiometric incorporation of each monomer into the polymer may well be invalid. As an alternative approach, the fluorophore contents of the probes were estimated from Beer–Lambert calibrations using an extinction coefficient of 150,000 l mol^{-1} cm^{-1} for the polymerised fluorophore, the same as that for the free Cy-3 fluorophore [29]. The Beer–Lambert plots for each probe were expressed as absorption verses concentration, from which the average molecular weight of a fluorophore-containing polymer segment was estimated. These average molecular weights are compared with those estimated for stoichiometric fluorophore incorporation in Table 2. The results suggest that in each case the relative amount of fluorophore incorporated in the final polymer was lower than that initially present in the reaction mixture. With the exception of PD40, which apparently contains relatively more fluorophores per polymer molecule than the other Polydyes, the ratio of molecular weights (based on extinction coefficients) in the Polydye series (20:40:60:80) correlate well with those calculated from the concentrations of fluorophores in the initial reaction mixtures (Table 2).

The fluorescence efficiency of fluorophores is known to change with their environment and may be reduced by fluorescence quenching at high concentrations. The possibility exists that non-random Cy-3 bis-amine incorporation might result in block co-polymer formation with high local concentrations of dye at certain regions of the polymer chain. Were this to occur with the Polydyes the fluorescence efficiency of PD20 would be lower than that of PD80 in

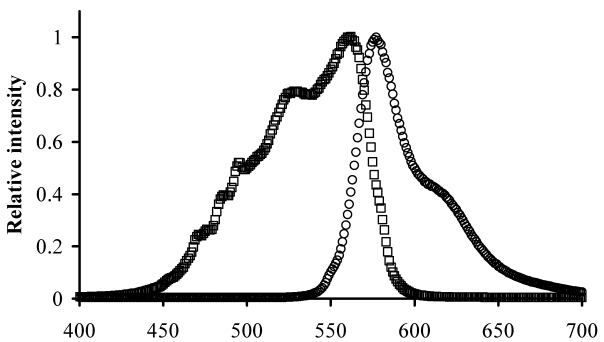

Fig. 4. Normalised excitation (□) and emission (○) spectra of PD20 measured in aqueous solution using an Aminco luminescence spectrofluorimeter (λ_{ex} = 540 nm, λ_{em} = 595 nm).

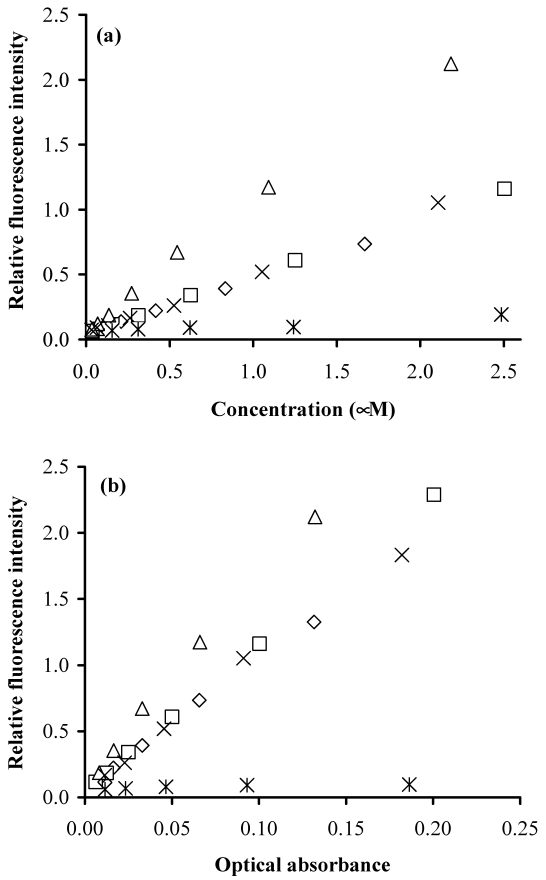

Fig. 5. Variation of relative fluorescence intensity of Polydyes 20 (×), 40 (△), 60 (□) and 80 (◇), and of the bis-amino Cy-3 monomer (∗), as a function of (a) the concentration of fluorophore containing polymer repeat unit, calculated on the assumption of stoichiometric fluorophore incorporation into the polymer, and (b) the optical absorbance of each Polydye sample. Absorbance measurements were taken at 553 nm in phosphate buffered saline at pH 7.4.

Table 2
Comparison of average molecular weights of fluorophore containing polymer segments and average relative ratios of fluorophores in polydyes; (A) calculated on the basis of stoichiometric incorporation of bis-amines in polydyes and (B) calculated from an extinction co-efficient of the polymerised fluorophores equivalent to that of unmodified Cy-3 in aqueous solution (150,000 l mol^{-1} cm^{-1})

Polydye	Relative molecular mass (A)	Relative molecular mass (B)	Relative ratio of fluorophores	
			(A)	(B)
20	6069	20,589	3.73	4.15
40	11,591	28,380	1.95	3.01
60	17,116	63,597	1.32	1.34
80	22,640	85,499	1.0	1.0

view of the four fold higher concentration of fluorophore monomers in the former, and hence the greater potential for block copolymer formation. Fig. 5(b) shows the relative fluorescence intensity of the different Polydyes and the Cy-3 bis-amine monomer plotted as a function of optical density. The identical behaviour of PD20 and PD80 demonstrates that there is no variation in fluorescence efficiency of these dyes over this range of low Cy-3 bis-amine incorporation and supports the view that the fluorescence behaviour of these materials is not influenced by dye association as would occur with non-random polymers.

3.3. Spectrophotometric titrations of the Polydye samples

The principal aim of the work was to establish a simple and reproducible technique for characterising the molecular conformation of hydrophobically modified polyelectrolytes of the type proposed. It has been established previously from potentiometric titration that poly(L-lysine *iso*-phthalamide) undergoes a hypercoiling transition between pH 4 and 5 [10]. Such a transition is characterised by the collapse of the polymer conformation into a tight coil that is stabilised by hydrophobic association. Thereby, the environment of an associated or copolymerised fluorophore is significantly changed and they are brought into closer proximity, effectively increasing their local concentration. Spectrophotometric titrations were first carried out for a mixture of poly(L-lysine *iso*-phthalamide) and free Cy-3 bis-amine at a fluorophore concentration of 0.26 mM (0.1 g l^{-1}), which gave the maximum emission intensity for this polymer/fluorophore combination. Fig. 6 shows an initial rise in relative fluorescence intensity of the non-polymerised fluorophore as the pH fell below the pK_a of the bis-amine groups at around pH 9.5, enabling increasing association of cationic fluorophore to the anionic polymer. A progressive increase in the relative fluorescence intensity is seen over the pH range 9.0–5.5 followed by a sharper increase in the range 5.5–5.0. The rapid increase is probably due to the onset of conformational collapse resulting in increased micro-viscosity and the development of local hydrophobic domains, both factors that would enhance fluorescence efficiency. There followed a rapid decrease in the relative fluorescence intensity in the pH range 5.25–4.3 which corresponds to the pH range over which poly(L-lysine *iso*-phthalamide) hypercoils. The relative fluorescence intensity of the free fluorophore then increased again following precipitation of the polymer at around pH 4.25. By contrast, spectrophotometric titration of the anionic bis-sulphonic acid Cy-3 derivative in the presence of poly(L-lysine *iso*-phthalamide) showed no similar increase in relative fluorescence intensity with pH until pH 5 (Fig. 7), when loss of charge on the polymer enables hydrophobic dye-polymer association. In this case, there was also no marked reduction in relative fluorescence intensity when the polymer conformation collapses.

When the Cy-3 bis-amine fluorophore was covalently bound within the backbone of the poly(L-lysine co-bis-amine-Cy-3 *iso*-phthalamide) broad similarities were observed in the variation of relative fluorescent intensity with solution pH (Fig. 8). No increase in relative fluorescence intensity was observed around the pK_a of the free fluorophore since the amine groups were converted to amide linkages. Similarly, there is little increase in the relative fluorescence intensity observed on precipitation of the polymer, since the fluorophores cannot be released into solution. These differences result from the conjugation of the fluorophore within the polymer.

Examination of the spectrophotometric titrations of the Polydye range shows an interesting trend (Fig. 8). As the amount of fluorophore is decreased the magnitude of the reduction in relative fluorescence intensity following polymer collapse decreases and is virtually absent in the case of the PD80 sample. This observation is consistent with the reduction in relative fluorescence intensity arising from the association of fluorophores on or within the polymer as it collapses. At lower degrees of substitution the occurrence of multiply labelled polymers is diminished, and the chance of intra-molecular fluorophore aggregation is thereby reduced.

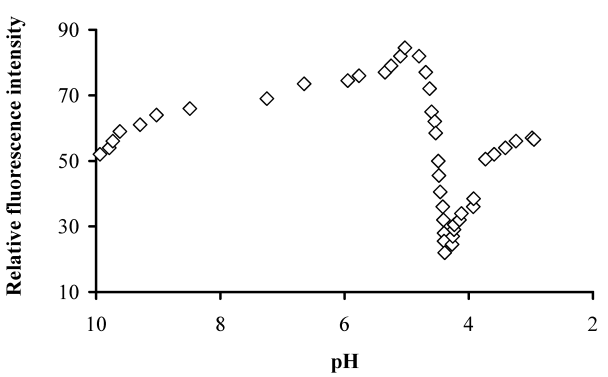

Fig. 6. Variation of relative fluorescence intensity of the Cy-3 bis-amino derivative in aqueous solution (0.26 mM) in the presence of 0.5 g l^{-1} poly(lysine *iso*-phthalamide) as a function of pH.

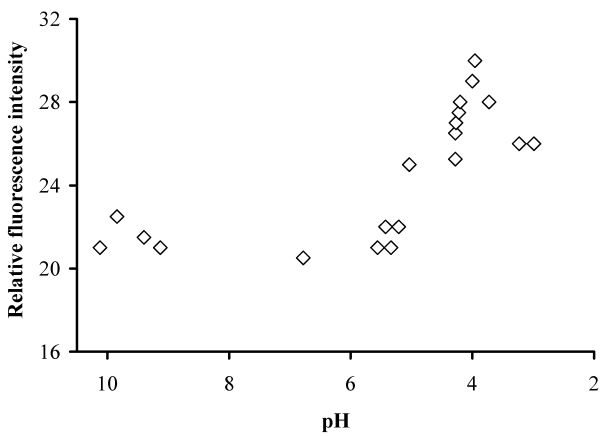

Fig. 7. Variation of relative fluorescence intensity of the Cy-3 bis-sulphonic acid derivative in aqueous solution (0.16 mM) in the presence of 0.5 g l^{-1} poly(lysine *iso*-phthalamide) as a function of pH.

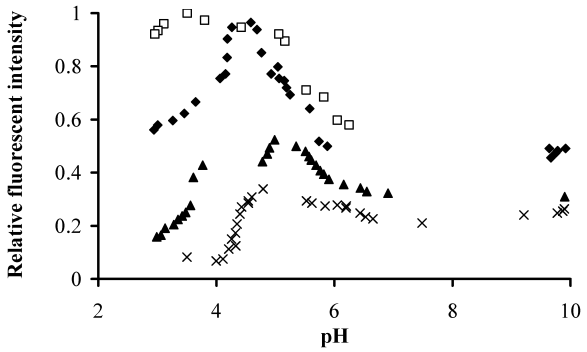

Fig. 8. Variation in relative fluorescence intensity of aqueous solutions (0.5 g l^{-1}) of PD20 (×), 40 (▲), 60 (◆), and 80 (□) titrated with 1.0N HCL delivered to an Aminco-SDP-125 spectrofluorimeter, equipped with a flow through cell, from a titration vessel via a peristaltic pump. Relative intensities normalised to PD80 with a fluorophore concentration of 0.58×10^{-5}M.

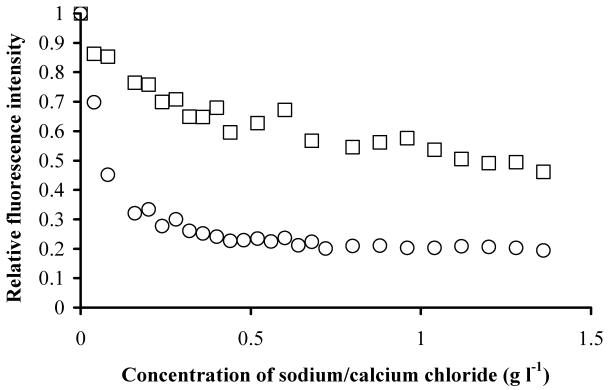

Fig. 9. Variation of relative fluorescence intensity of Cy-3 bis-amine derivative measured in a 0.5 g l^{-1} aqueous solution of poly(lysine *iso*-phthalamide) using a Cytofluor plate reader ($\lambda_{ex} = 535$ nm and $\lambda_{em} = 570$ nm) in the presence of sodium chloride (□) and calcium chloride (○).

The spectrophotometric titration of the PD80 sample is similar to that of the unlabelled poly(lysine *iso*-phthalamide) in the presence of the bis-sulphonic acid Cy-3 derivative, displaying only a small decrease in relative fluorescence intensity prior to precipitation.

Our observations are consistent with those of Rutkaite et al. [18], who noted similar pH dependent variations in the fluorescence behaviour of amphiphilic carbazolyl-containing poly methacrylate copolymers containing a range of fluorophore contents. Polymers with low fluorophore content (1% and less) showed increased fluorescence at low pH due to reduced exposure of the fluorophore to external quenchers in the coiled state. At higher degrees of fluorophore loading (9–54% (w/w)) this effect was masked by enhanced self-quenching due to hydrophobic association of the fluorophores within the collapsed polymer. If sufficiently high amounts of fluorophore were included (65 %) then no effect due to pH was seen, as the fluorophores were self-quenched at all pHs. Differences in the photophysical properties of the carbazolyl-containing poly methacrylate polymers and the cyanine containing poly (lysine *iso*-phthalamide) polymers investigated here, indicate that the latter undergo pH mediated self-quenching at much lower fluorophore loadings. For the cyanine containing materials quenching was observed at fluorophore to repeat unit ratios in the region of 2.1%.

Finally, we have observed that the relative fluorescence intensity of the mixed polymer/fluorophore samples were strongly dependent on the concentration of added salts. In the presence of sodium chloride, a near linear relationship between relative fluorescence intensity and salt concentration was observed whereas in the presence of divalent cations, such as calcium chloride a rapid drop in the relative fluorescence intensity was seen at low salt concentrations (Fig. 9). The decrease in relative fluorescence intensity at higher ionic strength presumably arises from changes in the polymer–fluorophore interaction due to increased charge shielding at higher salt concentrations [28]. In the case of the divalent calcium cation, the effect is enhanced by the ability of multivalent ions to coordinate several carboxyl groups. No such effect was observed with the fluorophore-conjugated Polydyes.

4. Conclusions

Conjugation of a bis-functional Cyanine fluorophore within the backbone of a hydrophobically modified polyamide, bearing weakly charged carboxyl groups pendant to the backbone, can be achieved by copolymerisation of the bis-amine functionalised fluorophore at the polymer synthesis stage. The fluorophore then acts as a reporter on the state of the polymer conformation, which can be manipulated either with solution pH or tonicity. The onset of polymer collapse is indicated by a rise in relative fluorescent intensity due to change in the environment of the polymer. The decrease in fluorescence output is dependent on the ratio of fluorophore to polymer, with high fluorophore containing polymers showing a decrease in relative fluorescence intensity by a factor of 4 from maximum emission at pH 4.8, and a minimum emission at pH 4.0 at the point of polymer precipitation. These values correlate well with the pH for onset of hypercoiling predicted by potentiometric titration for unmodified poly(L-lysine *iso*-phthalamide). Simple solutions of poly(L-lysine *iso*-phthalamide) and the bis-amino Cy-3 derivative behaved similarly with maximum relative fluorescence intensity at pH 4.9. Thus, conjugation of Cy-3 bis-amine within or with poly(L-lysine *iso*-phthalamide) allows the state of polymer conformation to be monitored simply by spectrophotometric titration. We are currently investigating the use of this system and a related Fluorescence Resonance Energy Transfer (FRET) system for use as novel bio-responsive signalling agents.

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