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PEGylation and aqueous solution behaviour of pH responsive poly(L-lysine *iso*-phthalamide)

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Abstract

The effect of PEGylation on the aqueous solution properties of a pH responsive pseudopeptide, poly(L-lysine *iso*-phthalamide), has been investigated using UV and fluorescence spectroscopy, ^{1}H NMR spectroscopy and dynamic light scattering. It was demonstrated that the level of PEGylation had a critical effect on the pH response of the parent polymer. When the degree of PEGylation was less than 23.4 wt% the modified polymer exhibited a transition from an expanded structure at high degrees of ionization to a compact hydrophobically stabilised structure at low degrees of ionization. The specific pH at which the conformational transition occurred was dependent on the degree of PEGylation. At levels of PEGylation in excess of 25.6 wt% the polymer no longer displayed this pH dependent conformation and existed in a micellar form (100–200 nm) over the whole range of ionization. Both linear and micellar forms of the pseudopeptide have applications in drug delivery.

Keywords: pH-responsive polymers; PEGylation; Micellisation

1. Introduction

Since Ringsdorf [1] first proposed a macromolecular prodrug model in 1975, there has been considerable research into the development of polymeric drug delivery systems. The versatility in polymer chemistry allows engineering of chemical structures to meet specific requirements and enhance the therapeutic potential of attached drugs through improved solubility, stability and targeting capability (both passive and active), with concomitant reduction of systemic side effects [2,3]. Key requirements of macromolecular delivery vehicles are their ability to evade the immune system and avoid renal clearance, thus prolonging circulatory half-life [3]. A common technique, used since the late 1970s, to impart bio-tolerance and increase molecular weight (above the renal threshold) is modification of the macromolecular carrier, with poly(ethylene glycol), commonly referred to as PEGylation [4,5].

Macromolecules generally enter cells by endocytosis when the plasma membrane surrounds the substance and buds off to form endosomes. These intracellular vesicles gradually acidify and fuse with enzyme laden lysosomes [6] limiting effective cytoplasmic delivery of enzyme-susceptible biomolecular drugs (e.g. DNA and proteins). Even if the payload is not degraded, compartmentalisation within endosomes/lysosomes can prevent a drug from reaching its intracellular target [7].

The pH-triggered conformational change of amphiphilic peptides on the surfaces of certain viruses play an important role in destabilising the endosomal membrane and allow release of viral contents into the cytoplasm [8]. This has spurred considerable interest in the use of pH-responsive polymers as membrane destabilising agents to efficiently release endocytosed macromolecules into the cytosol before they reach the lysosomes [9–12]. 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 structure, stabilised by hydrophobic association [13,14]. Such agents can disrupt

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the endosomal bilayer under slightly acidic conditions, pH 5.5–6.5, but be non-lytic at physiological pH 7.4 [15–18]. In particular, the pH mediated conformational transition and membrane destabilising effects of poly(methacrylic acid) [19], poly(ethylacrylic acid) [13,18] and poly(propylacrylic acid) [20] 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 [21]. It has been suggested that such polymers associate with the cell membrane by hydrogen bonding and/or hydrophobic association, and a reduction in pH leads initially to increased polymer–membrane interaction, and consequent disruption upon pH-triggered conformational change [19,22].

This laboratory has a long-standing interest in responsive biopolymer targeted drug delivery and medical imaging [18, 23–25]. A novel class of biocompatible metabolite-derived polyamides has been prepared by copolymerization of amino acids containing both α- and ω-amine groups and dicarboxylic acid chlorides [23]. pH dependant cell membrane lysis has been demonstrated [18] and the conformational behaviour characterised using a novel fluorescence labeling method [25]. A typical example is poly(L-lysine *iso*-phthalamide) [23]. We report here, the effects of PEGylation on the pH mediated conformational transition of this polymer. This study is the first systematic examination of manipulation of the hydrophilic/hydrophobic balance of a hydrophobically modified polyelectrolyte by PEGylation and its influence on the pH-response, which, together with the ongoing characterisation of the biological activity of these polymers provides important information on the fine control of molecular animation by PEGylation.

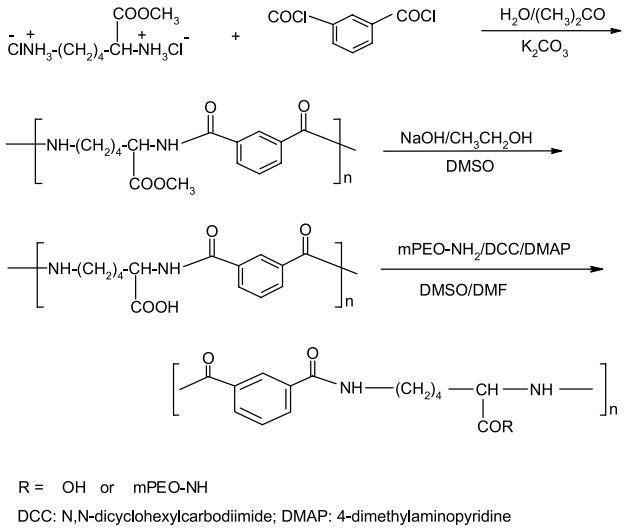
2. Experimental section

2.1. Materials

iso-Phthaloyl chloride, potassium carbonate, *N*,*N*′-dicyclohexylcarbodiimide (DCC), 4-dimethylaminopyridine (DMAP), methoxypoly(oxyethylene) amine (M_n = 4400 g mol^{-1}, mPEO-NH_2) and pyrene were obtained from Aldrich. L-lysine methyl ester dihydrochloride was purchased from Lancaster. Pyrene was recrystallised from ethanol three times. Dimethyl sulfoxide (DMSO) and acetone were dried using standard procedures [26]. All other reagents were used directly as received.

2.2. Synthesis

Poly(L-lysine *iso*-phthalamide)s with PEG side chains were synthesized according a procedure illustrated in Scheme 1. Poly(L-lysine *iso*-phthalamide) was prepared by hydrolysis of poly(lysine methyl ester *iso*-phthalamide) and converted to the sodium salt form according to the method



Scheme 1. PEGylation of poly(L-lysine *iso*-phthalamide).

of Eccleston et al. [23,27]. The polymer in neutral form was used for the structural characterisation and PEGylation, whilst the salt form was used to prepare aqueous solutions for spectrophotometric analysis (1.0 mg ml^{-1} unless specified otherwise). FTIR (acid form): 1718 (COOH stretch), 1627, 1527 cm^{-1} (amide band I and II). ^{1}H NMR (d$_6$-DMSO): δ (ppm) 1.20–1.88 (–CH_2–(CH_2)$_3$–CH–), 3.09–3.54 (–NH–CH_2–), 4.33–4.39 (–C*H*–NH–), 7.46–8.69 (–N*H*–, Ar–*H*). The ^{13}C NMR (d$_6$-DMSO) is shown in Fig. 1. The aqueous GPC analysis gave an M_n of 14,000 g mol^{-1} and an M_w of 24,000 g mol^{-1} with a polydispersity of 1.7.

Eight PEGylated polymers were synthesized, designated as PA-0.5, PA-1.0, PA-1.5, PA-2.0, PA-2.5, PA-3.0, PA-4.0 and PA-5.0, respectively, and their degrees of substitution were controlled by varying the molar ratios of [NH_2]/[COOH (Table 1). In a typical procedure poly(L-lysine *iso*-phthalamide) (3.00 g, 11.87 mmol [COOH]), DMAP (0.60 g, 20 wt% of the parent polymer) and mPEO-NH_2

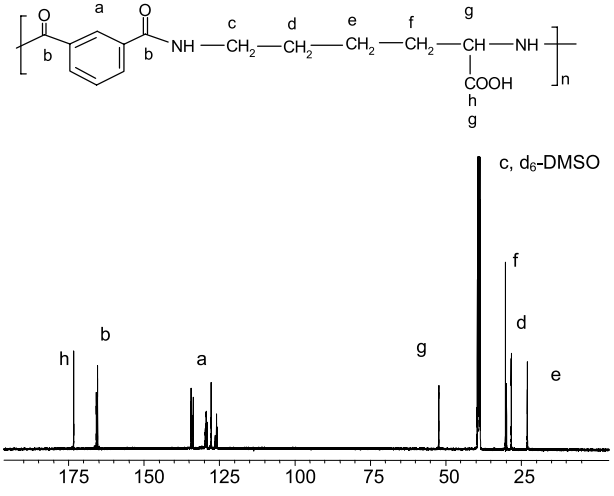


Fig. 1. ^{13}C NMR spectrum of poly(L-lysine *iso*-phthalamide) (in the acid form) in d$_6$-DMSO at room temperature.

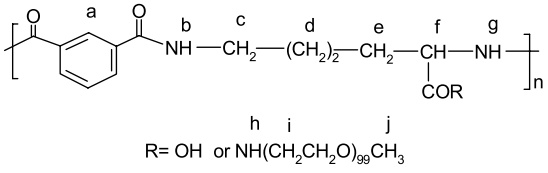
Table 1
Structural compositions of the PEGylated poly(L-lysine *iso*-phthalamide)s

	PA-0.5	PA-1.0	PA-1.5	PA-2.0	PA-2.5	PA-3.0	PA-4.0	PA-5.0
[NH_2]/[COOH]	0.5/100	1.0/100	1.5/100	2.0/100	2.5/100	3.0/100	4.0/100	5.0/100
DS[aa]	0.4	0.9	1.3	1.9	2.1	2.5	3.3	4.2
PEG wt%	6.6	12.5	17.4	23.4	25.6	29.2	35.3	40.9
M_n[b] (10^3)	14.9	15.9	16.9	18.2	18.8	19.7	21.6	23.6

[a] Defined as the number of the PEG side chains per 100 carboxylic acid groups and determined by ^{1}H NMR spectroscopy in d_6-DMSO at room temperature.
[b] Calculated based on PEG wt% and the M_n of poly(L-lysine *iso*-phthalamide) determined by light scattering.

(0.27 g, 0.054 mmol) were dissolved in anhydrous DMSO/DMF (1:3, 60 ml). DCC (0.45 g, 40 mol equiv of mPEO-NH_2) dissolved in anhydrous DMF (20 ml) was added dropwise over a period of 1/2 h and the reaction stirred at room temperature for 48 h. The solution was then dialysed against deionised water to remove the organic solvents and the solid impurities removed by vacuum filtration. Low molecular weight impurities were removed by diafiltration (Millipore, MWCO 5 kDa) against four volumes of deionised water followed by concentration and lyophilisation (pH adjusted to ~7 using NaOH) to give PA-0.5 as a fine white powder. Persistent PEG impurities were removed by Soxhlet extraction with ethyl acetate for 24 h, as indicated by aqueous GPC analysis.

Neutralised polymers were prepared by acidification of their solutions to pH ~3.0, and isolated by filtration or lyophilisation, depending on the degree of PEGylation. The degree of PEGylation was calculated from the ^{1}H NMR spectrum recorded in d_6-DMSO, e.g. Fig. 2. Polymers with increased degrees of PEGylation were prepared analogously.



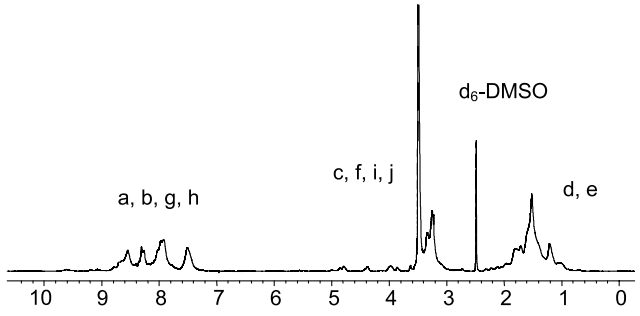


Fig. 2. Typical ^{1}H NMR spectrum of PEGylated poly(L-lysine *iso*-phthalamide) (PA-2.5, 25.6 wt% PEG in the acid form) in d_6-DMSO at room temperature.

2.3. Characterization

FTIR spectra were recorded on a Thermo Nicolet Nexus FT-IR spectrophotometer. ^{1}H and ^{13}C NMR spectra (500 MHz) were obtained on a Bruker Advance 500 spectrometer at room temperature. The T_gs of the polymers in the acid form were determined on a Perkin–Elmer DSC 7 at a heating rate of 10 K min^{-1} in a nitrogen atmosphere. The molecular weight of poly(L-lysine *iso*-phthalamide) was determined by Viskotek Ltd on an aqueous GPC system consisting of 2×30 cm ViscoGEL GMPW columns equipped with a triple detector TDA 302 (low angle light scattering, refractive index and viscometer) with 0.01 M potassium dihydrogenphosphate buffer adjusted to pH 7.0 as eluant at a flow rate of 0.6 ml min^{-1}. The M_n, in combination with the degree of PEGylation, was used for calculation of the molecular weights of PEGylated polymers. The separation of PEGylated polymers from unreacted PEG was monitored on a Viscotek aqueous GPC 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 eluant and a flow rate of 1 ml min^{-1}.

2.4. Aqueous solution behaviour

The conformations of the parent polymer and its PEGylated derivatives were investigated in aqueous solution over a range of pHs by fluorescence spectroscopy, UV spectroscopy and dynamic light scattering at room temperature.

2.4.1. Fluorescence spectroscopy

The emission (λ_{ex}=335 nm) and excitation (λ_{em}=390 nm) spectra of pyrene dissolved in aqueous solutions of the polymers were recorded on a Jobin Yvon Huriba Fluoro Max-3 spectrofluorimeter at right angle geometry with slit widths of 1 nm for emission and 5 nm for excitation.

A stock solution of pyrene in absolute methanol (1.0 mM) was used to prepare an aqueous solution at 6×10^{-7} M by adding 600 μl of the methanolic solution into 1 l deionised water [28]. Solutions of freely soluble polymers, PNa, PA-0.5, PA-1.0, PA-1.5 and PA-2.0, were prepared by mixing 50 mg of polymer with 50 ml of the aqueous pyrene

solution. Polymers with higher degrees of PEGylation, PA2.5–PA5.0, required heat or a co-solvent (methanol) to achieve dissolution. In this case, 50 mg of polymer was dissolved in 20 ml of methanol/water (50/50) and 30 μl of 1 mM methanolic pyrene added. The methanol was removed by dialysis against deionised water for 48 h and the volume adjusted to 50 ml with deionised water. Stock solutions were allowed to equilibrate for 48 h and sample solutions were prepared with pHs ranging from 3 to 10 by addition of 0.3 N HCl or 0.3 N NaOH. All samples were allowed to equilibrate for 1 h before recording spectra.

2.4.2. Turbidimetry

The pH-induced phase separation of the PEGylated polymers in aqueous solution was investigated on a Shimadzu UV-160A spectrophotometer, by measuring the optical density at 480 nm of the polymer solutions as a function of pH. Solutions were prepared as for fluorescence measurements.

2.4.3. Dynamic light scattering

The hydrodynamic diameters of the micelles in aqueous solutions of the highly PEGylated polymers were measured using a PDDLS/Batch dynamic light scattering platform (Precision Detectors) equipped with a PD2000DLS dynamic light scattering detector. The measurements were conducted using a diode laser of 800 nm at a scattering angle of 90°. The polymer samples were filtered though 0.45 μm pore size filters before use.

3. Results and discussion

3.1. Synthesis

PEG side chains were introduced onto poly(L-lysine *iso*-phthalamide) using standard DCC mediated coupling chemistry [29]. Careful control over reaction conditions, principally concentration of the polymer, was required to avoid gelation of the system due to the formation of intermolecular anhydrides [30]. Purification of the PEGylated polymer derivatives by dialysis alone proved ineffective, even with a relatively large MWCO diafiltration membrane (30,000 g mol^{-1}). Complete removal of unreacted PEG, as verified by GPC (see Fig. 3), was achieved by Soxhlet extraction with ethyl acetate. The low efficiency of PEG removal by dialysis has also been noted by Winblade et al. in the preparation of poly-L-lysine-graft-poly(ethylene glycol) [31].

The PEGylated polymers were white powders in both neutralised and ionised states, and those in the acid form were characterised with ^{1}H NMR spectroscopy in d_6-DMSO, from which the degrees of PEGylation were determined. A typical ^{1}H NMR spectrum of the PEGylated polymers in d_6-DMSO is shown in Fig. 2, and the compositions of the graft polymers listed in Table 1. The

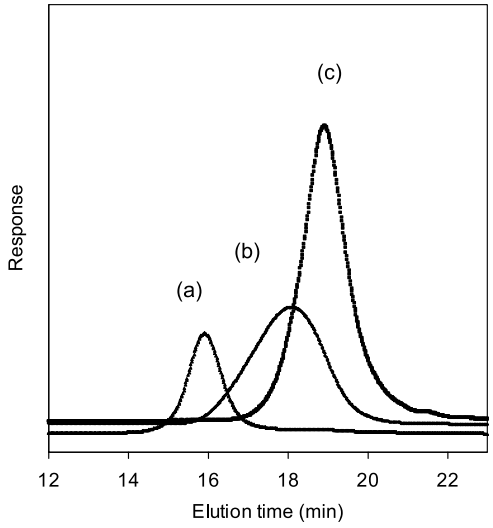


Fig. 3. Aqueous GPC traces obtained at room temperature in 0.1 N $NaNO_3$ methanol/water (15% methanol), (a) PA-4 (35.3 wt% PEG) in the salt form, (b) poly(L-lysine *iso*-phthalamide) in the salt form and (c) mPEO-NH_2.

degrees of PEGylation were calculated based on a M_n for mPEO-NH_2 of 4400 g mol^{-1} without taking account of its polydispersity (1.1). They are expressed as the numbers of PEG grafts per 100 carboxylic acid groups (DS^a) and the weight percentage of the PEG side chains (PEG wt%). It can be seen from Table 1 that a small rise in DS^a leads to a large increase in PEG wt% due to the large molecular weight of the PEG.

3.2. Thermal properties

The DSC thermogram of poly(L-lysine *iso*-phthalamide) in the acid form reveals a glass transition at ~433 K in the temperature range of 293–523 K. The high T_g reflects the contribution of extensive inter- and intramolecular hydrogen bonding. All the PEGylated polymers investigated here exhibit a single T_g indicating that the polymer backbone and the PEG side chains are miscible. It can be seen in Table 2 that the T_g values of the PEGylated polymers increase progressively with increasing degree of PEGylation up to ~459 K at a degree of PEGylation of ~34.4 wt% then decrease with further PEGylation. This may result from a balance of two opposing effects of PEGylation on the glass transition temperature of the resultant polymers, increasing T_g due to hydrogen bonding between the PEG side chains and carboxylic acid groups [32] and decreasing T_g by side chain plasticization.

3.3. Aqueous solution behaviour of poly(L-lysine iso-phthalamide)

The amphiphilic structure of poly(L-lysine *iso*-phthalamide) leads to pH-mediated conformational transitions in water, driven by a balance of electrostatic repulsion and hydrophobic associative interactions [18]. It is readily

Table 2
Variation of the glass transition temperatures (T_g) as a function of PEG wt% of PEGylated poly(L-lysine *iso*-phthalamide)

	Poly(L-lysine *iso*-phthalamide)	PA-0.5	PA-1.0	PA-1.5	PA-2.0	PA-2.5	PA-3.0	PA-5.0
PEG wt%	0	6.6	12.5	17.4	23.4	25.6	35.3	40.9
T_g (K)	433	418	427	433	435	442	459	429

soluble in the ionised state, but tends to precipitate out upon neutralisation. The pH-induced phase separation is manifested by a precipitous decrease in the transmittance due to formation of polymer aggregates (Fig. 4). The pH at onset of precipitation of the polymer solution, defined as the onset of decrease in solution transmittance, is ~5.5. It can be seen from Fig. 4 that a 10 fold dilution of the polymer causes a shift in the pH at onset of precipitation to ~3.1, which indicates that the pH-induced phase separation is also concentration dependant.

Pyrene is a hydrophobic fluorescent probe used extensively in studies of the aqueous solutions of amphiphilic polymers due to the sensitivity of its photophysical properties towards the polarity of its microenvironment [33]. Transferring pyrene from a polar to nonpolar environment results in a pronounced reduction in the intensity ratio of the first to the third vibronic peak in its emission spectra (I_1/I_3), coupled with a red shift from 332.5 to 338 nm in the low-energy band of the L_a transition in excitation spectra. Thus, the intensity ratios of I_1/I_3 and I_{338}/I_{333} in the fluorescent spectra of pyrene have been used routinely to express the polarities of its microenvironment [34].

I_1/I_3 and I_{338}/I_{333} for pyrene were determined as a function of pH in the aqueous solution of poly(L-lysine *iso*-phthalamide) at 1.0 mg ml^{-1}. As seen in Fig. 5, I_1/I_3 is almost constant at ~1.78 at pH≥5.8. This value is close to that of pyrene in water at room temperature [35], indicating the location of pyrene in a polar microenvironment irrespective of the presence of the polymer. As the solution pH is reduced from ~5.8 to ~4.7 there is a rapid decrease in I_1/I_3 suggesting a significant increase in the hydrophobicity of the pyrene microenvironment. There is a further gradual reduction in I_1/I_3 until pH~3.0 when there is a sudden increase in I_1/I_3, which may be due to the release of some pyrene into the water resulting from the polymer precipitation. A change of the pyrene microenvironment, from hydrophilic to hydrophobic with decreasing solution pH, is also reflected by the shift of the maximum wavelength corresponding to the low-energy transition in excitation spectra from 333 to 338 nm (spectra not shown). I_{338}/I_{333} is almost constant, ~0.52, at pH≥5.8, but increases markedly to ~1.22 upon reduction of pH to ~4.7.

The pronounced pH-mediated change in the polarity of pyrene microenvironment can be ascribed to a pH-dependant conformational change of poly(L-lysine *iso*-phthalamide). At sufficiently high pH most of the carboxylic acids are ionised and the polymer adopts an extended chain conformation in water, driven by the mutual electrostatic repulsions of the weakly charged groups, and the pyrene is solvated by the aqueous medium. As the pH decreases, neutralisation of some of the carboxylic acids takes place and hydrophobic interactions drive the collapse of the polymer with a concomitant formation of hydrophobic microdomains. Pyrene is highly hydrophobic and is believed to partition preferentially into the hydrophobic domains. This has been well validated by the use of pyrene to identify the hydrophobic microdomains in micellar solutions in water [33,34]. Similar variation of I_1/I_3 has been reported with pH for poly(methacrylic acid) [35,36]

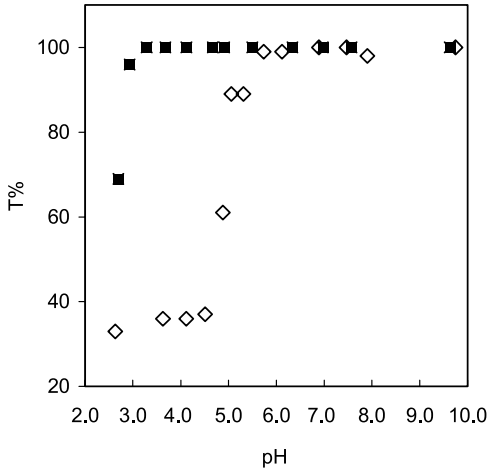


Fig. 4. Variation of transmittance of the aqueous solutions of poly(L-lysine *iso*-phthalamide) at different pH, measured at λ=480 nm, polymer concentration 1.0 mg ml^{-1} (■) and 0.1 mg ml^{-1} (◇), respectively.

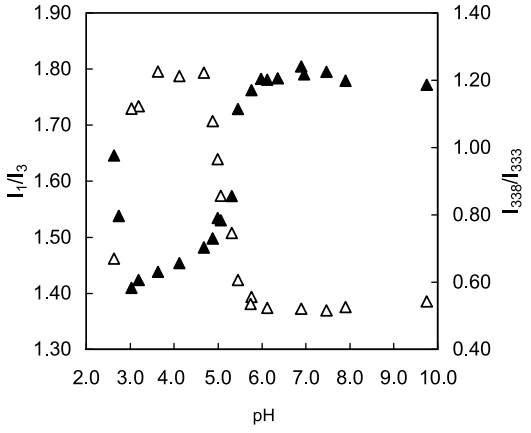


Fig. 5. Variations of I_1/I_3 (▲) and I_{338}/I_{333} (△) as a function of pH in the emission and excitation spectra of pyrene in aqueous poly(L-lysine *iso-ph*thalamide) at 1 mg ml^{-1}.

and its derivatives [19], and with temperature for thermo-responsive poly(*N*-alkylacrylamide)s [37]. These polymers are known to undergo pH or temperature induced conformational transition between expanded and globular (hyper-coiled) state [38,39]. Thus, the conformational transition of poly(L-lysine *iso*-phthalamide), as revealed by the ratios I_1/I_3 and I_{338}/I_{333}, takes place over the pH range of ~5.8 to ~4.7 (Fig. 5). The pH of onset for significant hydrophobic associations, defined here as the point where the I_1/I_3 starts to decrease or the I_{338}/I_{333} starts to increase, is ~5.8.

These results are in a good agreement with previous spectrophotometric analysis of cyanine fluorophores electrostatically associated to poly(L-lysine *iso*-phthalamide) where the emission intensity of fluorophores was shown to be strongly influenced by the pH-mediated conformation transition of the polymer. Fluorescence decreases on polymer collapse due to self-quenching as the fluorophores are brought into closer proximity. On polymer precipitation some fluorophores are released back into solution as indicated by an increase in the observed fluorescence [25].

Added insight into the pH-mediated conformation of this polymer in water was obtained by comparison of the pyrene emission spectra of dilute polymer solutions. The solutions were allowed to equilibrate for 48 h following dilution before the spectra were recorded. The variation in I_1/I_3 with pH is strongly dependant on polymer concentration, indicating that intermolecular interactions may play an important role. There is less distinctive decrease in I_1/I_3 upon reduction of pH together with relatively higher I_1/I_3 values at acidic pH upon dilution of the initial polymer concentration by 3 and 10 fold compared with the parent polymer solution (Fig. 6). This indicates that at these concentrations as the pH decreases the polymer tends to form less compact microdomains since the pyrene is in a less hydrophobic environment. The relatively high value of I_1/I_3 (~1.80) and lack of significant variation with pH in the polymer solution at 0.01 mg ml^{-1}, indicates that a relatively open conformation is adopted by the polymer throughout the pH range studied. This suggested that upon reduction of pH intramolecular hydrophobic association alone may generate insufficiently hydrophobic domains and that the pyrene remains exposed to the aqueous environment.

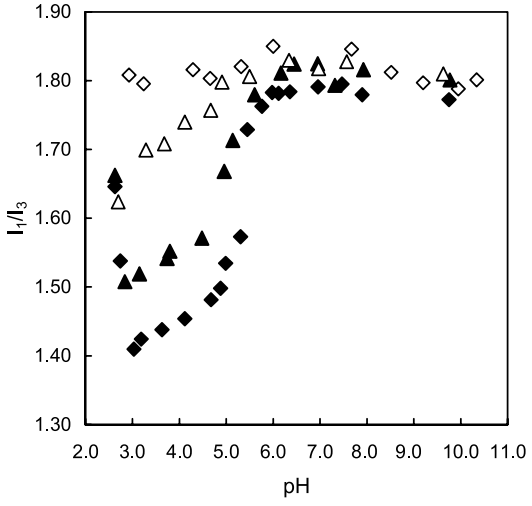


Fig. 6. Variations of I_1/I_3 as a function of pH in the emission spectra of pyrene dissolved aqueous poly(L-lysine *iso-ph*thalamide) at 1.0 mg ml^{-1} (◆), 0.3 mg ml^{-1} (▲), 0.1 mg ml^{-1} (△) and 0.01 mg ml^{-1} (◇).

3.4. Aqueous solution behaviour of PEGylated poly(L-lysine iso-phthalamide)

Turbidity tests were conducted on the aqueous solutions of the modified polymers (1.0 mg ml^{-1}) to determine the effect of PEGylation on the pH-induced phase separation of these polymers. The pH of onset of precipitation of these polymer solutions are shown as a function of degree of PEGylation in Table 3. A shift of the pH at onset of precipitation towards low pH is observed with increasing PEG content, indicating an increasing stability of these aqueous solutions at low pH as a result of PEGylation. No phase separation was observed in the solution of polymer with 40.9 wt% PEG within the pH range of 2–11.

The solution properties of the PEGylated polymers were also investigated with fluorescence spectroscopy using pyrene as a probe. Both the emission and excitation spectra of the pyrene solubilised in the aqueous solution of each polymer were recorded as a function of pH, and the corresponding I_1/I_3 and I_{338}/I_{333} calculated. Since the variation of I_{338}/I_{333} with pH showed a similar trend to the variation of I_1/I_3 with pH, the following discussion will focus on the I_1/I_3 results.

The variation of I_1/I_3 with pH could be broadly separated into two categories, shown in Fig. 7(a) and (b). For polymers with <23.4 wt% PEG the plot of variation of I_1/I_3 with pH is a similar shape to that of the parent polymer. This shape of titration curve is indicative of a pH-driven conformational transition from an extended conformation, dominant by electrostatic repulsion, to a relatively compact structure, stabilised by hydrophobic interactions, in these polymer solutions, i.e. the pH-driven conformational change present in the poly(L-lysine *iso*-phthalamide) solution is retained to some extent over this range of degree of PEGylation. By contrast, the I_1/I_3 ratios in solutions of polymers with > 25.6 wt% PEG are relatively low and do not vary much over the whole pH range examined (Fig. 7(b)), implying the absence of this pH-mediated conformational change. Thus the maximum degree of PEGylation for retention of this pH-mediated conformational transition is between ~23.4 and ~25.6 wt%, which can be more clearly seen in Fig. 8 where I_1/I_3 of polymer solutions at pH 7.5 and pH 4.0 is plotted as a function of PEG wt%. For <23.4 wt% PEG there are marked differences in I_1/I_3 of the polymer solutions at pH 7.5 and pH 4.0, indicative of two conformations that are significantly different in polarity which can be switched by varying pH. However, increasing the amount of PEG to ~25.6 wt% leads to the disappearance of the pH-driven

Table 3
Effect of PEGylation on the pH at onset of precipitation of the aqueous polymer solutions at 1.0 mg ml^{-1}

	Poly(L-lysine *iso*-phthalamide)	PA-0.5	PA-1.0	PA-1.5	PA-2.0	PA-2.5	PA-3.0	PA-4.0
PEG wt%	0	6.6	12.5	17.4	23.4	25.6	35.3	40.9
pH at onset of precipitation	5.5±0.2	4.9±0.1	5.2±0.2	4.7±0.2	4.1±0.4	4.1±0.3	4.4±0.2	3.3±0.2

transition, as indicated by the fairly close values of I_1/I_3 of polymer solutions at these two pHs. No significant change was observed with further rise in the degree of PEGylation. The fact that the I_1/I_3 ratios of the solutions of polymer with PEG contents >25.6 wt% at pH 7.5 is lower than or close to those of the solutions of polymers with relatively low PEG contents at pH 4.0, points to the existence of hydrophobic domains throughout the pH range examined. Given the fact that the I_1/I_3 values of pyrene in aqueous solution of mPEO-NH_2 at the same concentration, which do not vary significantly with pH, are close to that in water (data not shown), these hydrophobic domains are composed of the collapsed polymer backbones, surrounded by the hydrophilic PEO side chains.

The pHs of onset of hydrophobic association and the pH range over which the conformational transition takes place for the PEGylated polymers and the parent polymer are summarised in Table 4. In each case, the pH of onset of hydrophobic association is higher than the corresponding pH at onset of precipitation determined by turbidity (Fig. 4), especially for polymers with 17.4 and 23.4 wt% of PEG, indicating the formation of hydrophobic microdomains well before polymer precipitation.

It is interesting to note that incorporation of appropriate amounts of hydrophilic PEG side chains leads to a pronounced shift of the pH of onset of hydrophobic association to higher pH. The pH for onset of hydrophobic association for solutions of polymer with 17.4 and 23.4 wt% PEG are ~6.8 and ~7.3, respectively, compared to 5.9 for the parent polymer. This effect is accompanied by a widening on the pH range over which the transition occurs. In contrast, solutions of polymers with 6.6 and 12.5 PEG wt% show a slight decrease in pH of onset of hydrophobic association to 5.5 and 5.6, respectively. Hydrophobic modification has been use to modulate the pH of onset of hydrophobic association of poly(acrylic acid) [38] and poly(methacrylic acid) [39–41] to high pH. Gaspar et al. [42] also found PEG side chains enhanced hydrophobic interactions of copolymers of acrylic acid and octadecyl methacrylate. Poe et al. [32] noted that coupling

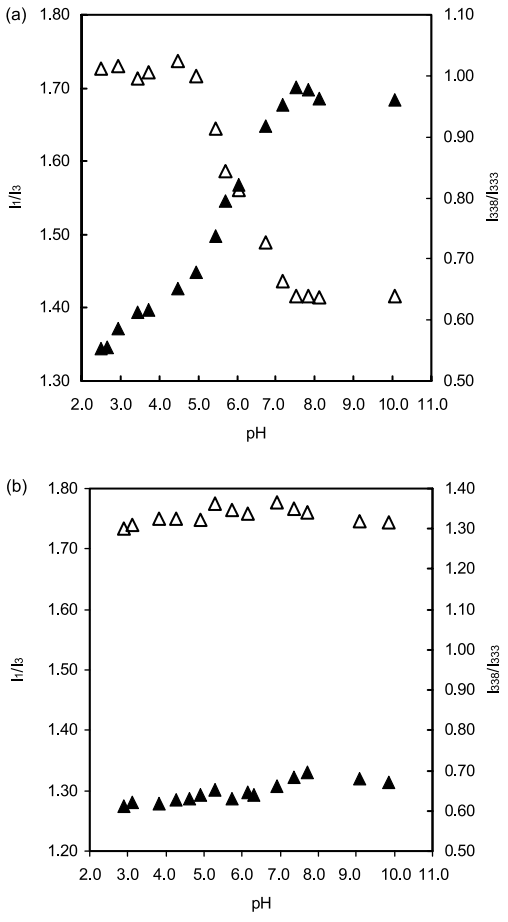


Fig. 7. Variations of I_1/I_3 (▲) and I_{338}/I_{333} (△) as a function of pH in the emission and excitation spectra of pyrene dissolved in (a) aqueous PA-2 (23.4 wt% PEG) at 1.0 mg ml^{-1} and (b) aqueous PA-3 (29.2 wt% PEG) at 1.0 mg ml^{-1}.

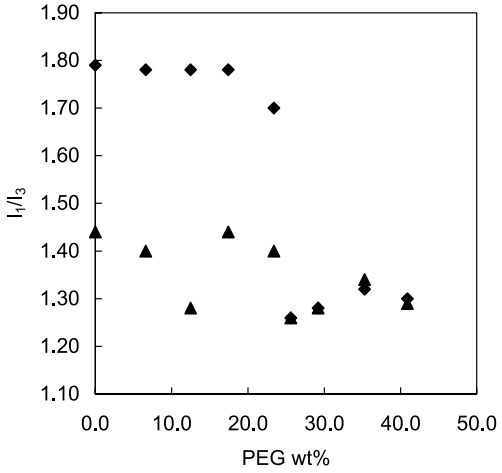


Fig. 8. Effects of PEGylation on the I_1/I_3 in the emission spectra of pyrene dissolved in aqueous PEGylated polymers (1.0 mg ml^{-1}) at pH 7.5 (◆) and pH 4.0 (▲).

Table 4
The pH values of onset of hydrophobic association and pH ranges for conformational change of PEGylated poly(L-lysine *iso*-phthalamide)s

	Poly(L-lysine *iso*-phthalamide).	PA-0.5	PA-1.0	PA-1.5	PA-2.0	PA-2.5
PEG wt%	0	6.6	12.5	17.4	23.4	25.6
pH at onset of hydrophobic association	5.9±0.1	5.5±0.1	5.6±0.2	6.8±0.4	7.3±0.2	N/A
pH range	5.8–4.7	5.4–4.3	5.4–4.6	7.1–4.9	7.5–4.9	N/A

Methoxy-PEG (5000 Da) to poly(methacrylic acid) caused a marked increase in the pK_a values at a degree of ionisation of 0.35 compared to either the non conjugated poly-(methacrylic acid) or mixtures of the two homopolymers. They postulated that intramolecular hydrogen bonding contributed to the stability of the collapsed coils produced at low pH. Similar effects are known to occur in hydrogel systems containing PEG grafted weakly acidic polymers [43]. The work presented here indicates that PEGylation can be used to controllably tailor the pH dependant hydrophobic association of a hydrophobically modified weakly charged polyelectrolyte.

For aqueous solutions of polymers with >25.6 wt% PEG side chains, hydrophobic domains are present over the whole pH range studied as indicated by the fluorescence results discussed above. The inherent amphiphilic nature of those materials, leads to the formation of micellar assemblies in which the polymer backbones collapse into the hydrophobic core with the PEG side chains forming the hydrophilic corona. This view is supported by ^{1}H NMR and dynamic light scattering measurements. Fig. 9 shows the ^{1}H NMR spectra of the polymer with 35.3 wt% PEG in the sodium salt form in D_2O and d_6-DMSO at room temperature. In contrast to the spectrum in d_6-DMSO, where distinct peaks corresponding to the polymer backbone and the PEG side chains are evident, the spectrum in D_2O is similar to that of pure PEG, with the resonance of the polymer backbone markedly attenuated. This indicates the polymer backbone is in a state of dehydration within a compact micellar core [44]. Hydrodynamic radii of the micelles formed in these solutions at pH 7.0 are in the range 10–50 nm (data not shown). A detailed study of these polymeric micelles will be presented in a forthcoming paper.

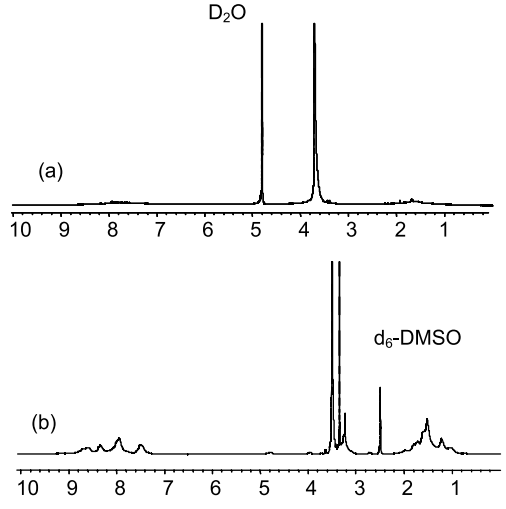


Fig. 9. Comparison of ^{1}H NMR spectra of the PEGylated poly(L-lysine *iso*-phthalamide) (PA-4, 35.3 wt% PEG, sodium salt form) in (a) D_2O and (b) d_6-DMSO at room temperature.

The presence of micellar structures in these polymer solutions may explain the difficulty in re-dissolving samples isolated by lyophilisation. Preservation of the micellar structures during freeze drying leaves tightly packed hydrophobic cores that may be responsible for the low solubility in water. The polymers can be redissolved into an organic solvent such as methanol and diluted into water. The organic solvent can then be removed by diafiltration.

In the above discussions, the effect of the decrease in charge density on the polymer conformation, as a consequence of PEGylation, is not taken into account. However, although the PEG content was varied markedly from 6.6 to 40.9 wt%, the corresponding number of unreacted carboxylic acids per 100 carboxylic acids, as calculated from the DS[a] in Table 1, decreases only from 99.6 to 95.8.

4. Conclusions

Poly(L-lysine *iso*-phthalamide) was PEGylated with mPEO-NH_2 (M_n 4400 g mol^{-1}) in the presence of DCC and DMAP, and the influence of degree of PEGylation on the physiochemical properties of the conjugated polymers, in particular the pH responses, was investigated. It was found that the degree of PEGylation significantly affects the pH-responsive behaviour of the polymer conjugates in terms of the pH of onset of hydrophobic association and the pH range over which the conformational transition takes place. Thus low levels of PEGylation can be used to tailor the onset of pH response in a hydrophobically modified polyelectrolyte whilst higher levels promote the formation of stable micelles. Both pH responsive soluble and micellular forms can be employed in novel drug delivery applications. The PEGylated systems are expected to show enhanced biocompatibility and a study on the pH dependent haemolytic activity and cytotoxicity will be published separately.

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