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Synthesis and pH-responsive properties of pseudo-peptide containing hydrophobic amino acid grafts

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Publication Details

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Abstract
Pseudo-peptidic polymers have been synthesised by grafting l-valine (PV), l-leucine (PL) and l-phenylalanine (PP) onto the pendant carboxylic acid moieties of a pH-responsive polyamide, poly(l-lysine isophthalamide). The pH-responsive aqueous solution properties of PV-75, PL-75 and PP-75 with a stoichiometric degree of substitution of 75 mol% have been compared with those of the parent poly(l-lysine isophthalamide) using UV-visible and fluorescence spectroscopy. At low concentrations (≤0.1 mg mL⁻¹), the grafted polymers displayed pH-dependent conformation. The pH at the onset of hydrophobic association (pHh) and the pH range over which association occurred varied significantly between the different amino acid grafts. The pHh values of PV-75, PL-75 and PP-75 at 0.025 mg mL⁻¹ were 6.2, 7.0 and 7.2, respectively. Increasing concentration enhanced intermolecular aggregation. A bis-functional Cy5 derivative, incorporated within the backbones of poly(l-lysine isophthalamide) (polyCy5) and PP-75 (PDP-75), was demonstrated to act as a fluorescence reporter on the state of polymer conformation and aggregation. The intracellular trafficking of PDP-75, examined by confocal microscopy, indicates potential applications of the grafted polymers in drug delivery and medical imaging. © 2009 The Royal Society of Chemistry.

Keywords
grafts, responsive, containing, ph, peptide, pseudo, properties, synthesis, hydrophobic, amino, acid

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Synthesis and pH-responsive properties of pseudo-peptides containing hydrophobic amino acid grafts

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Pseudo-peptidic polymers have been synthesised by grafting L-valine (PV), L-leucine (PL) and L-phenylalanine (PP) onto the pendant carboxylic acid moieties of a pH-responsive polyamide, poly(l-lysine isophthalamide). The pH-responsive aqueous solution properties of PV-75, PL-75 and PP-75 with a stoichiometric degree of substitution of 75 mol% have been compared with those of the parent poly(l-lysine isophthalamide) using UV-visible and fluorescence spectroscopy. At low concentrations (≤0.1 mg mL⁻¹), the grafted polymers displayed pH-dependent conformation. The pH at the onset of hydrophobic association (pH₅₃) and the pH range over which association occurred varied significantly between the different amino acid grafts. The pH₅₃ values of PV-75, PL-75 and PP-75 at 0.025 mg mL⁻¹ were 6.2, 7.0 and 7.2, respectively. Increasing concentration enhanced intermolecular aggregation. A bis-functional Cy5 derivative, incorporated within the backbones of poly(l-lysine isophthalamide) (polyCy5) and PP-75 (PDP-75), was demonstrated to act as a fluorescence reporter on the state of polymer conformation and aggregation. The intracellular trafficking of PDP-75, examined by confocal microscopy, indicates potential applications of the grafted polymers in drug delivery and medical imaging.

Introduction

The realisation by De Duve et al.¹ that cellular endocytosis is useful for lysosomotropic drug delivery, and Ringsdorf’s concept of a polymer-drug conjugate model,² have stimulated great interest in polymer-mediated drug delivery. Intracellular delivery of macromolecules is commonly limited by trafficking via endosomes to lysosomes, where they may be degraded by enzymes³,⁴ or accumulated unproductively.⁵ Fusogenic peptides facilitate efficient viral infection by mediating membrane disruption upon acidification in endosomes, and this has been exploited in the use of inactivated viruses and viral peptides for drug delivery.⁶,⁷ Nevertheless, high-potency vectors of viral origin raise safety concerns and are costly to manufacture. Attention has therefore been paid to the development of biomimetic polymers for use as non-viral vectors.

Amphiphilic polymers containing weakly ionisable carboxyl groups and hydrophobic alkyl side chains can mimic the structure of viral peptides.⁸ Such polymers display a change of conformation from extended chains to collapsed, hydrophobically-stabilised globular structures upon reduction of pH below their pKₐ ranges.⁹ This change leads to increased interaction of polymers with cell membranes and subsequent membrane disruption.¹⁰⁻¹⁻² The pH-mediated change of conformation depends principally on the balance between the hydrophobic interaction associated with hydrophobic moieties and the electrostatic repulsion resulting from charged carboxyl groups.¹³ The hydrophobicity of pendant hydrophobic groups is one of the factors that influences the conformational and membrane-disruptive behaviour of pH-responsive polymers.¹⁴⁻¹⁶,¹⁸ Thus, the vinyl polymers poly(α-methylacrylic acid), poly(α-ethylacrylic acid) and poly(α-propylacrylic acid) undergo conformational change at progressively higher pH values corresponding to increasing endosomal membrane lysis activity.¹⁷⁻¹⁹

Eccleston et al.²⁰⁻²² described a biodegradable, metabolite-derived polyamide, poly(l-lysine isophthalamide), displaying a pH-dependent conformational change²³⁻²⁵ and limited cell membrane lytic capacity over a low pH range (4.6–5.0).²⁶ This pseudo-peptide comprises a hydrophobic backbone and pendant carboxyl groups, but lacks pendant hydrophobic groups present in fusogenic peptides and poly(α-alkylacrylic acid)s. Recently we have conducted hydrophobic modification of poly(l-lysine isophthalamide) with three different amino acids: L-valine and L-leucine with alkyl groups of different lengths and L-phenylalanine with an aromatic group. The effects of modification on the pH modulated cell membrane-disruptive activity and intracellular trafficking of the grafted polymers have been demonstrated and presented elsewhere.²⁷ In this work, the effects of the different pendant hydrophobic groups on the pH-responsiveness of polymer conformation and aggregation are investigated to elucidate the polymer-mediated cell membrane destabilisation. Incorporation of a bis-carboxyl chloride derivative of the cyanine fluorophore Cy5 within the polymer backbone allows spectrophotometric determination of pH-dependent polymer conformation and aggregation. The subcellular localisation of the fluorescent grafted polymer and its ability to release endocytosed materials into the cytoplasm of mammalian cells are

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further evaluated and potential applications in drug delivery and medical imaging are discussed in this article.

**Experimental**

**Materials**

Isophthaloyl chloride, potassium carbonate, N,N′-dicyclohexylcarbodiimide (DCC), 4-dimethylaminopyridine (DMAP), N,N′-dimethylformamide (DMF), triethylamine, pyrene and calcein were purchased from Sigma-Aldrich (Dorset, UK). L-lysine methyl ester dihydrochloride, disodium hydrogen phosphate, sodium dihydrogen phosphate, trisodium citrate and citric acid were obtained from Fisher (Loughborough, UK). L-phenylalanine methyl ester hydrochloride, L-leucine methyl ester hydrochloride and L-valine methyl ester hydrochloride were purchased from Alfa Aesar (Heysham, UK). Pyrene was purified by recrystallisation from ethanol three times. Dimethyl sulfoxide (DMSO) was dried using a standard procedure. All other reagents were used directly as received. RPMI-1640 medium (without L-glutamine) was purchased from Invitrogen (Paisley, UK). Foetal bovine serum (FBS), Dulbecco’s phosphate buffered saline (D-PBS), penicillin–streptomycin solution (10 000 units mL⁻¹ penicillin and 10 mg mL⁻¹ streptomycin), L-glutamine solution (200 mM) and trypsin–EDTA solution (0.5 g L⁻¹ porcine trypsin and 0.2 g L⁻¹ EDTA-4Na) were obtained from Sigma-Aldrich (Dorset, UK).

**Synthesis of poly(L-lysine isophthalamide)s grafted with hydrophobic amino acids**

Poly(L-lysine isophthalamide) (Mw = 35 700, Mθ = 17 900, polydispersity = 1.99), prepared according to the method of Eccleston et al., was grafted with L-valine, L-leucine and L-phenylalanine (designated as PV, PL and PP series polymers, respectively) according to the procedure illustrated in Fig. 1a. Three grafted polymers PV-75, PL-75 and PP-75 are reported here. The number “75” signifies the stoichiometric molar percentage of amino acids relative to the pendant carboxylic acid groups along the backbone of poly(L-lysine isophthalamide). In a typical procedure, poly(L-lysine isophthalamide) (3 g, 10.87 mmol [COOH]), L-phenylalanine methyl ester·HCl (1.76 g, 8.15 mmol [NH₂]), triethylamine (2.4 molar equivalents of L-phenylalanine methyl ester·HCl) and DMAP (0.6 g, 20 wt% of poly(L-lysine isophthalamide)) were dissolved in anhydrous DMSO–DMF (1 : 3 v/v, 60 mL), to which was added dropwise DCC (5.05 g, 3 molar equivalents of L-phenylalanine methyl ester·HCl) in anhydrous DMF (20 mL). The reaction was allowed to proceed at room temperature for 60 h. Solid impurities were removed by vacuum filtration and lyophilised to a fine white powder. PV-75 and PL-75 were prepared similarly. FTIR (acid form): 3294 cm⁻¹ (N–H str), 1720 cm⁻¹ (amide I), 1528 cm⁻¹ (amide II), 1463 cm⁻¹ (C=C str), 1382 cm⁻¹ (amide III), 1159 cm⁻¹ (C–O str), 1088 cm⁻¹ (C–O str). The degrees of grafting of PV-75, PL-75 and PP-75 were calculated from their ¹H-NMR spectra recorded in d₆-DMSO (Fig. 2 and Table 1).

**Synthesis of poly(L-lysine isophthalamide) equivalent polydye grafted with L-phenylalanine**

Poly(L-lysine co-bis-carboxyl-Cy5 isophthalamide), denoted as polyCy5, was polymerised from isophthaloyl chloride-bis-carboxyl chloride derivative of the cyanine fluorophore Cy5 (30 : 1 mol/mol) and L-lysine methyl ester·2HCl according to the method of Eccleston et al. This poly(L-lysine isophthalamide) equivalent “polydye” was grafted with L-phenylalanine using a similar procedure to that for PP-75 (designated as PDP-75, Fig. 1b). The degree of grafting of PDP-75 was calculated from its ¹H-NMR spectrum recorded in d₂-DMSO (Table 1).

**Structural characterisation**

FTIR spectra were recorded on a Nicolet Nexus FTIR spectrometer equipped with an ATR “Goldengate™” sampling system (Thermo Fisher Scientific, USA). ¹H-NMR spectra were obtained in d₂-DMSO on a Bruker Advance 500 MHz spectrometer (Bruker Biospin GmbH, Germany) at room temperature. The molecular weight and polydispersity of poly(L-lysine
isophthalamide) were determined on an aqueous GPC system (Viscotek, UK), which consists of 2 × 30 cm ViscoGEL GMPW columns equipped with a VE3580 refractive index detector, using 0.1 M NaNO₃ containing 15 wt% methanol as an eluent at a flow rate of 1.0 mL min⁻¹. The GPC system was calibrated with polyethylene glycol standards.

Preparation of buffer solutions

0.1 M phosphate buffers in the pH range of 6.20–8.10 were prepared by mixing 0.2 M NaH₂PO₃ and 0.2 M Na₂HPO₃ aqueous solutions followed by two-fold dilution with deionised water. Final pH values were measured using a pH meter. 0.1 M citrate buffers in the pH range of 3.52–8.10 were prepared similarly by mixing 0.2 M citric acid and 0.2 M trisodium citrate aqueous solutions.

**Turbidimetry**

A turbidity test was used to determine the pH values at which phase separation occurred.²⁴,³⁰ Polymer solutions were prepared with the buffers in the pH range of 3.52–8.10 and allowed to equilibrate for 48 h. The optical densities of the solutions at 480 nm were measured on a Spectronic UV1 spectrophotometer (Thermo Fisher Scientific, USA).

**Fluorescence spectroscopy**

300 µL of 1.0 mM pyrene in absolute methanol was added to 500 mL of the buffers to give a pyrene concentration of 6 × 10⁻⁷ M with pH ranging from 3.52 to 8.10. Poly(t-lysine isophthalamide), PV-75, PL-75 and PP-75 were dissolved in deionised water and diluted 20-fold with appropriate pyrene-containing buffers. The polymer solutions were allowed to equilibrate for 48 h in the dark before use. The emission spectra (λₑₓ = 335 nm) of pyrene²⁹ dissolved in the polymer solutions were recorded on a SPEX FluoroMax-3 spectrofluorimeter (Jobin Yvon Horiba, UK) at right-angle geometry. The emission spectra (λₑₓ = 639 nm) of polyCy5 and PDP-75 dissolved in the buffer solutions in the absence of pyrene were recorded similarly.

**Cell culture**

HeLa adherent epithelial cells (human cervical cancer cells) were grown in RPMI-1640 medium supplemented with 10% (v/v) FBS, 2 mM l-glutamine, 100 U mL⁻¹ penicillin and 100 µg mL⁻¹ streptomycin unless specified otherwise. The HeLa cells were trypsinised using trypsin–EDTA and maintained in a humidified incubator with 5% CO₂ at 37 °C.

**Laser scanning confocal microscopy**

Calcein (a membrane-impermeable fluorophore) was used as a tracer molecule to monitor the effect of the polymers on internalised vesicles. 2 mL of HeLa cells (1–2 × 10⁵ cells mL⁻¹) were seeded into a 35 mm glass-bottom culture dish (MatTek, USA) and cultured for 24 h in an incubator at 37 °C. The spent medium was removed and replaced with 2 mL of serum and phenol red free medium containing 0.22 mM filter-sterilised

### Table 1 Composition measurements for poly(t-lysine isophthalamide), PV-75, PL-75, PP-75 and PDP-75

<table>
<thead>
<tr>
<th></th>
<th>Poly(t-lysine isophthalamide)</th>
<th>PV-75</th>
<th>PL-75</th>
<th>PP-75</th>
<th>PDP-75</th>
</tr>
</thead>
<tbody>
<tr>
<td>[NH₂]/[COOH] (%)</td>
<td>0</td>
<td>75</td>
<td>75</td>
<td>75</td>
<td>75</td>
</tr>
<tr>
<td>DS/mol%</td>
<td>0</td>
<td>62.1</td>
<td>61.2</td>
<td>63.2</td>
<td>60.0</td>
</tr>
<tr>
<td>Weight percentage of amino acid grafts/wt%</td>
<td>0</td>
<td>21.4</td>
<td>23.1</td>
<td>28.1</td>
<td>27.1</td>
</tr>
<tr>
<td>Mₙ (× 10⁷)</td>
<td>17.9</td>
<td>22.8</td>
<td>23.3</td>
<td>24.9</td>
<td>24.6</td>
</tr>
</tbody>
</table>

² Stoichiometric molar percentage of amino acids (t-valine, t-leucine and t-phenylalanine) relative to the carboxylic acid groups along the backbone of poly(t-lysine isophthalamide) or polyCy5.
³ Degree of substitution, defined as the number of amino acid grafts per 100 carboxylic acid groups and determined by ¹H-NMR in d₆-DMSO at room temperature.
⁴ Calculated based on the weight percentage of amino acid grafts (wt%) and the Mₙ of poly(t-lysine isophthalamide) determined by GPC.
PDP-75 at the described concentration and 2 mg mL\(^{-1}\) calcein (these solutions were titrated to pH 7.4 using 0.1 M NaOH). After being incubated for 30 min at 37 °C, the cells were washed three times with D-PBS and further incubated with serum and phenol red free medium for 3.5 h at 37 °C. The cells were then removed from the incubator and imaged on a FluoView™ FV300 inverted laser scanning confocal microscope (Olympus, Japan) within 15 min. The labelled Cy5 within PDP-75 was excited using a 633 nm laser and the Cy5 emission at 680 nm was collected. Calcein was excited using a 488 nm laser and the calcein emission at 535 nm was collected.

Results and discussion

Synthesis

The carboxylic acid groups of poly(l-lysine isophthalamide) were grafted with l-valine, l-leucine and l-phenylalanine at the same stoichiometric degree of substitution of 75% using DCC as a coupling agent and DMAP as a hyperacylation catalyst.\(^{31,32}\) While keeping the backbone length of poly(l-lysine isophthalamide) constant, the structures of amino acid grafts were varied, thereby manipulating the molecular weights, physico-chemical properties and biological activities of grafted polymers.

The grafted polymers in the acid forms were characterised by \(^1\)H-NMR spectroscopy in d\(_6\)-DMSO, as shown in Fig. 2. The ratios of the integral 0.67–1.02 ppm to the integral 7.31–8.95 ppm (Fig. 2a), the integral 0.63–0.97 ppm to the integral 7.31–8.95 ppm (Fig. 2b) and the integral 6.90–7.31 ppm to the integral 7.31–8.95 ppm (Fig. 2c) were used to determine the degrees of grafting of PV-75, PL-75 and PP-75, respectively. They are expressed as the numbers of amino acid grafts per 100 carboxylic acid groups along the backbone of poly(l-lysine isophthalamide) (DS, mol%) and the weight percentages of the grafts (wt%) as shown in Table 1. The molecular weights of PP-75, PL-75 and PV-75 were calculated from the \(M_b\) of poly(l-lysine isophthalamide) and degrees of grafting, and are also shown in Table 1. The degree of grafting of PDP-75 (mol% and wt%) was determined similarly, as shown in Table 1. Due to the low level of incorporation of Cy5 and the degraded resolution in the proton spectra,\(^{31,32}\) it was difficult to determine the amount of Cy5 loading and Cy5 distribution for PDP-75.

Phase behaviour in aqueous solution

The effects of modification with hydrophobic amino acids on the phase behaviour of poly(l-lysine isophthalamide) in aqueous solution were investigated by measuring the optical densities at 480 nm of the polymer solutions as a function of pH. As shown in Fig. 3, the pH value at the onset of precipitation (pH\(_p\)) for the aqueous solution of poly(l-lysine isophthalamide), determined as the pH value at which the optical transmittance started to decrease, can be modulated by amino acid grafting. Compared to the parent polymer, PV-75, PL-75 and PP-75 all exhibited higher pH\(_p\) values, which ranked in the order: poly(l-lysine isophthalamide) < PV-75 < PL-75 ≈ PP-75. This ranking reflects the increased hydrophobicity of polymers resulting from the conjugation of hydrophobic amino acids with increasing hydrophobicity. Normalised to a scale of 0 for glycine and 100 for the most hydrophobic amino acid among the 20 natural amino acids, the hydrophobicity indices of valine, leucine and phenylalanine at pH 7.0 are 76, 97 and 100, respectively,\(^{33}\) which are close to those at acidic pH 2.0 (79, 100 and 92, respectively).\(^{34}\)

The work described here is consistent with a previous study which showed that increasing the hydrophobicity of monomers can increase the precipitation pH.\(^{31}\) The pH-mediated phase separation of aqueous polymer solutions was concentration dependent. At 1.0 and 0.5 mg mL\(^{-1}\) the polymers showed a sharp full in optical transmittance upon acidification due to the precipitation of polymer molecules. At the lower concentration of 0.1 mg mL\(^{-1}\), the polymers exhibited a less abrupt phase change. At 0.025 mg mL\(^{-1}\) no visible phase separation was observed within the pH range examined (data not shown). The pH\(_v\) values of poly(l-lysine isophthalamide), PV-75, PL-75 and PP-75 at concentrations ranging from 0.025 to 1.0 mg mL\(^{-1}\) are summarised in Table 2, indicating that increasing concentration enhanced intermolecular aggregation.

Fluorescence characterisation using pyrene as a probe

The changes in the ratio of intensities of the first to the third vibronic peak in the emission spectrum of pyrene (denoted as \(I_1/I_3\)), induced by environmental polarity changes,\(^{29}\) were employed to investigate the effects of polymer concentration and hydrophobic amino acid grafts on the pH-induced hydrophobic association of polymers. Fig. 4 shows the changes of \(I_1/I_3\) as a function of pH in the solutions of poly(l-lysine isophthalamide), PV-75, PL-75 and PP-75 at the concentrations ranging from 0.025 to 1.0 mg mL\(^{-1}\). As shown in Fig. 4a, the pH at the onset of hydrophobic association (pH\(_b\)), defined here as the pH value at which \(I_1/I_3\) started to decrease, was ~5.3 for poly(l-lysine isophthalamide) at 1.0 mg mL\(^{-1}\). At pH ~5.3, the \(I_1/I_3\) ratio was almost constant at ~1.87, characteristic of pyrene molecules in a hydrophilic aqueous environment at room temperature.\(^{48}\) A rapid decrease in \(I_1/I_3\) was observed when the pH was reduced further before reaching a plateau at pH 4.3. This indicates an increasingly hydrophobic environment of pyrene in the pH range of 5.3–4.3. The pronounced pH-mediated change in the polarity of pyrene environment can
be ascribed to a pH-dependent change of polymer conformation from extended chains to collapsed structures with a concomitant formation of hydrophobic domains\textsuperscript{24,36} and formation of aggregates\textsuperscript{37} upon acidification. With decreasing concentration of poly(L-lysine isophthalamide), the variation of $I_1/I_3$ with pH was less pronounced. At 0.025 mg mL$^{-1}$, the $I_1/I_3$ ratio remained high at 1.80–1.90 over the pH range studied. This concentration-dependent change indicates that intermolecular interactions may play an important role and that, at dilute polymer concentrations, intramolecular hydrophobic association alone may generate insufficiently hydrophobic domains, resulting in exposure of pyrene to the aqueous environment.\textsuperscript{24}

The effects of concentration on the pH-mediated solution behaviour of the grafted polymers depend on the nature of side chains. The variations in $I_1/I_3$ for pyrene dissolved in the aqueous solutions of PV-75, PL-75 and PP-75 are displayed in Fig. 4b, 4c, and 4d, respectively. At 1.0 and 0.5 mg mL$^{-1}$, the $I_1/I_3$ values in the presence of three modified polymers were low (1.15–1.45) and almost independent of pH. These observations contrast markedly to the rapid drop over a narrow pH range for poly(L-lysine isophthalamide), indicating the presence of pyrene within hydrophobic domains over the pH range examined. At 0.025, 0.05 and 0.1 mg mL$^{-1}$, all three grafted polymers displayed a gradual decrease in $I_1/I_3$ with pH. In the case of PP-75 (Fig. 4d), the $I_1/I_3$ ratios at neutral pH were considerably lower than that of pyrene in water, pointing to a less hydrophilic environment of pyrene resulting from the less expanded polymer conformation.\textsuperscript{36} Upon acidification, a further progressive decrease in the apparent polarity of the pyrene environment was observed due to enhanced hydrophobic association of the polymer, which led to the envelopment of pyrene molecules within the resulting hydrophobic domains.

### Table 2

The pH values at the onset of precipitation (pH$_p$) of poly(L-lysine isophthalamide), PV-75, PL-75 and PP-75 at the concentrations ranging from 0.025 to 1.0 mg mL$^{-1}$ in 0.1 M buffers

<table>
<thead>
<tr>
<th>C/mg mL$^{-1}$</th>
<th>Poly(L-lysine isophthalamide)</th>
<th>PV-75</th>
<th>PL-75</th>
<th>PP-75</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.025</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>0.05</td>
<td>4.2 ± 0.2</td>
<td>4.2 ± 0.4</td>
<td>4.3 ± 0.2</td>
<td>5.1 ± 0.1</td>
</tr>
<tr>
<td>0.1</td>
<td>4.4 ± 0.2</td>
<td>4.8 ± 0.2</td>
<td>5.2 ± 0.2</td>
<td>5.2 ± 0.2</td>
</tr>
<tr>
<td>0.5</td>
<td>4.6 ± 0.1</td>
<td>4.9 ± 0.1</td>
<td>5.5 ± 0.1</td>
<td>5.4 ± 0.1</td>
</tr>
<tr>
<td>1.0</td>
<td>4.7 ± 0.1</td>
<td>4.9 ± 0.1</td>
<td>5.6 ± 0.2</td>
<td>5.5 ± 0.1</td>
</tr>
</tbody>
</table>

**Fig. 4** Variations in $I_1/I_3$ as a function of pH in the emission spectra of pyrene dissolved in the aqueous solutions of (a) poly(L-lysine isophthalamide), (b) PV-75, (c) PL-75 and (d) PP-75 at 0.025 (○), 0.05 (◇), 0.1 (□), 0.5 (●) and 1.0 mg mL$^{-1}$ (■) in 0.1 M buffers.
As summarised in Table 3, at 0.025 mg mL$^{-1}$, the pH$_h$ values increased in the order: PV-75 (pH$_h$ = 6.2) < PL-75 (pH$_h$ = 7.0) < PP-75 (pH$_h$ = 7.2), together with a broadening of the corresponding pH range over which association occurred. At 0.05 mg mL$^{-1}$, the same trend was observed for three modified polymers with higher pH$_h$ values and further broadening of the pH ranges. The enhanced hydrophobic association of PL-75 and PP-75 compared to the parent polymer and PV-75 is ascribed to the increased hydrophobicity resulting from the introduction of amino acid grafts (l-leucine and l-phenylalanine) with higher hydrophobicity indices. It is consistent with the observation that peptides with higher hydrophobicity show stronger association in aqueous solution. Others have noted that poly(ε-methylacrylic acid), poly(ε-ethylacrylic acid) and poly(ε-propylacrylic acid) with increasingly hydrophobic pendant alkyl groups change conformation at pH 5.0, 5.5, and 6.5 respectively. PP-75 exhibited a higher pH$_h$ than PL-75 although l-phenylalanine has a similar hydrophobicity index to l-leucine, which could be due to the enhanced capacity of PP-75 to form hydrophobic domains resulting from interactions between aromatic rings on the polymer backbone and those on the l-phenylalanine grafts, and between l-phenylalanine moieties. It has been reported that the π–π interactions between the benzyl cores of the micelles formed by polyethylene glycol-b-poly(ɛ-benzyl-l-aspartate) and the aromatic rings of anticancer drugs (e.g. doxorubicin) can contribute to the stabilisation of micellar structures with increased amounts of drug loading. This ranking of hydrophobic association activity amongst poly(ɛ-lysine isophthalamide), PV-75, PL-75 and PP-75 correlates well with their increasing abilities to haemolyse cell membranes. This supports the statement that increasingly hydrophobic and compact structures, which result from significantly enhanced hydrophobic association upon reduction of pH, could cause increased polymer–membrane interactions and subsequent higher levels of membrane disruption. The pH$_h$ values of the grafted polymers were considerably higher than their pH$_p$ values (Table 2 and 3), indicating that upon acidification the hydrophobic domains were formed well before precipitation, consistent with the observations for PEGylated polymers and methacrylic acid copolymers.

Fluorescence characterisation of fluorescent polymers

Incorporation of a low level of the bis-functional cyanine fluorophore Cy5 within the polymer backbone was conducted to further explore the effect of pH on polymer conformation and aggregation. The variations in the emission intensity (integrated over the range 500–900 nm) of the cyanine fluorophore within polyCy5 and PDP-75 are shown as a function of pH in Fig. 5. At 1.0 mg mL$^{-1}$, the emission intensity of polyCy5 was strongly influenced by pH. The intensity was almost constant upon acidification until pH 5.4. With further acidification to pH 4.6, a gradual increase in intensity occurred, which can be due to the protection of Cy5 from external quenchers offered by the increased micro-viscosity and formation of local hydrophobic domains. At pH 4.1, an abrupt reduction in intensity was caused by self-quenching as the fluorophores came into close proximity within compact hydrophobic cores of collapsed macromolecular structures or intermolecular aggregates formed from hydrophobic association upon reduction of pH. At 0.7 mg mL$^{-1}$ polyCy5 displayed a similar pH-mediated change in emission intensity with lower intensity values. This is in agreement with previous observations on the related polyCy3 systems. For PDP-75 at 1.0 mg mL$^{-1}$, which contains an equivalent content of fluorophore Cy5 to polyCy5 at 0.7 mg mL$^{-1}$, the integrated emission intensity remained close to or even lower than that of polyCy5 in the state with compact hydrophobic cores. This suggests the existence of compact hydrophobic domains formed by PDP-75 throughout the pH range examined.

Table 3 The pH values at the onset of hydrophobic association (pH$_h$) and pH ranges for association of poly(ɛ-lysine isophthalamide), PV-75, PL-75, and PP-75 at the concentrations ranging from 0.025 to 1.0 mg mL$^{-1}$ in 0.1 M buffers

<table>
<thead>
<tr>
<th>C/mg mL$^{-1}$</th>
<th>Poly(ɛ-lysine isophthalamide)</th>
<th>PV-75</th>
<th>PL-75</th>
<th>PP-75</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pHHpH range</td>
<td>pH$_h$</td>
<td>pH range</td>
<td>pH$_h$</td>
</tr>
<tr>
<td>0.025</td>
<td>N/A</td>
<td>6.2 ± 0.2</td>
<td>6.3–4.1</td>
<td>7.0 ± 0.1</td>
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<tr>
<td>0.05</td>
<td>N/A</td>
<td>6.4 ± 0.1</td>
<td>6.5–3.8</td>
<td>7.1 ± 0.1</td>
</tr>
<tr>
<td>0.1</td>
<td>4.3 ± 0.3</td>
<td>6.4 ± 0.2</td>
<td>6.4–3.8</td>
<td>6.5 ± 0.1</td>
</tr>
<tr>
<td>0.5</td>
<td>4.4 ± 0.2</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>1.0</td>
<td>5.3 ± 0.2</td>
<td>N/A</td>
<td>N/A</td>
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Fig. 5 pH-mediated changes of the emission intensity (integrated over 500–900 nm, $\lambda_{ex} = 639$ nm) of polyCy5 at 1.0 (▼) and 0.7 mg mL$^{-1}$ (○) and PDP-75 at 1.0 mg mL$^{-1}$ (△) in 0.1 M buffers. 0.7 mg mL$^{-1}$ polyCy5 contains an equivalent content of fluorophore Cy5 to 1.0 mg mL$^{-1}$ PDP-75. The intensity was normalised against the emission maximum of polyCy5 at 1.0 mg mL$^{-1}$ (pH 4.6).
A pronounced pH-mediated redshift in the wavelength of the fluorescence emission maximum ($\lambda_{\text{max}}$) was observed for PDP-75 at the lower concentration of 0.025 mg mL$^{-1}$ (Fig. 6). This indicates increased hydrophobic association of PDP-75 upon acidification, resulting in an increased local concentration of the fluorophores in close proximity. Such proximity enhanced π–π stacking between the Cy5 segments and de-excitation of fluorophores prior to emission.\(^{43,44}\) Fig. 6 shows that the trend of redshift with pH for PDP-75 is in agreement with the pH-dependence of $I_3/I_1$ for PP-75 at the same concentration, further confirming that grafting poly(l-lysine isophthalamide) with hydrophobic amino acids can induce significant pH-mediated hydrophobic association, leading to a pH-dependent formation of hydrophobic domains.

**Subcellular localisation of PDP-75 and release of endocytosed calcein**

The pH-, concentration- and time-dependent cell membrane-disruptive activity of poly(l-lysine isophthalamide), PV-75, PL-75 and PP-75 has been reported recently using a haemolysis model. The grafted polymers can be non-haemolytic at pH 7.4, but mediate significant membrane disruption at endosomal pH (5.0–6.8) in the order: PP-75 > PL-75 > PV-75 and poly(l-lysine isophthalamide).\(^{27}\) The most active PP-75 was 35-fold more lytic on a molar basis than the membrane-lytic peptide melittin, and has been demonstrated to release endocytosed materials significantly into the cytoplasm of mammalian cells.\(^{27}\) Here, the conformation-dependent fluorescence behaviour of the pH-responsive Cy5-containing PDP-75 grafted polymer, which shared the similarity on molecular structure and hydrophobic association with PP-75, has prompted an investigation on its intracellular trafficking to explore the application of fluorescent polymers in drug delivery and medical imaging. As shown in Fig. 7a, no fluorescence was detected in HeLa cells without the treatment of PDP-75 or calcein. When the cells were incubated with 2 mg mL$^{-1}$ calcein alone, the membrane-impermeable fluorophore was restricted within intracellular vesicles appearing as bright punctate spots (Fig. 7b), consistent with constitutive endocytosis of the external medium.\(^{45}\) HeLa cells in Fig. 7c and d were imaged after being treated with 0.025 mg mL$^{-1}$ PDP-75 and 2 mg mL$^{-1}$ calcein for 30 min and further incubated for 3.5 h. As seen in Fig. 7c, the punctate Cy5 spots suggest that PDP-75 was internalised by endocytosis, which is often upregulated in tumours,\(^{46}\) and trapped within intracellular vesicles, whilst the diffuse staining pattern is indicative of release of the fluorescent polymer into the cytoplasm.\(^{27,45}\) Although Fig. 7d shows a number of bright calcein spots in most areas of the cells, some diffuse fluorescence was noted spreading over the cells, suggesting the release of endocytosed calcein from intracellular vesicles into the cytoplasm.\(^{27,45}\) The two images in Fig. 7e and f show cells treated with 1.0 mg mL$^{-1}$ PDP-75 and 2 mg mL$^{-1}$ calcein. The significantly enhanced diffuse staining of both Cy5 (Fig. 7e) and calcein (Fig. 7f) indicates that increasing PDP-75 concentration induced increased endosomal/lysosomal membrane disruption and subsequent cytoplasmic release of endocytosed calcein. Thus, by tailoring the pH response of the fluorescent grafted

![Fig. 6](image-url)  
**Fig. 6** Fluorescence characterisation of the fluorescent polymer PDP-75 compared with fluorescence characterisation of PP-75 using pyrene as a probe. The $I_3/I_1$ ratios in the emission spectra of pyrene dissolved in 0.025 mg mL$^{-1}$ PP-75 (●), and the wavelengths corresponding to maximum fluorescence emission ($\lambda_{\text{max}}$) for its equivalent PDP-75 polydye at 0.025 mg mL$^{-1}$ (○), are measured as a function of pH.

![Fig. 7](image-url)  
**Fig. 7** Intracellular localisation of PDP-75 polydye (c, e) and calcein dye (b, d, f) in HeLa cells. (a) Cells without the treatment of PDP-75 or calcein. (b) Cells were treated with 2 mg mL$^{-1}$ calcein. (c) and (d) Cells were treated with both 0.025 mg mL$^{-1}$ PDP-75 and 2 mg mL$^{-1}$ calcein. (e) and (f) Cells were treated with both 1.0 mg mL$^{-1}$ PDP-75 and 2 mg mL$^{-1}$ calcein. Images were acquired with an Olympus FluoView™ FV300 confocal unit (×60 magnification) at 3.5 h after 30 min of uptake.
polymers to the pH range within endosomes, their ability to release endocytosed materials into the cytoplasm, together with the change in optical signal, might permit potential applications in drug delivery and medical imaging.

Conclusions

Poly(t-lysine isophthalamide) has been grafted with the same stoichiometric degree of substitution (75 mol%) of hydrophobic amino acids, t-valine, t-leucine and t-phenylalanine, to tailor the intramolecular balance between hydrophilic and hydrophobic moieties. It has been shown that the pH-responsive behaviour of poly(t-lysine isophthalamide) can be modulated by varying the length of the alkyl side groups and introducing pendant aromatic groups. At low polymer concentrations ($\leq 0.1 \text{ mg mL}^{-1}$), the pH at the onset of hydrophobic association increased significantly following the order: poly(t-lysine isophthalamide) < PV-75 < PL-75 < PP-75. This was accompanied by a marked broadening of the pH range over which association occurred. At sufficiently high concentrations ($\geq 0.5 \text{ mg mL}^{-1}$), the grafted polymers formed compact hydrophobic domains over the pH range examined. Incorporation of Cy5-dye moieties within the backbone of the grafted polymers allows rapid spectrophotometric determination of polymer conformation and aggregation and offers potential drug delivery and medical imaging applications.

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References