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pH-Responsive, Lysine-Based, Hyperbranched Polymers Mimicking Endosomolytic Cell-Penetrating Peptides for Efficient Intracellular Delivery

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ABSTRACT

The insufficient delivery of biomacromolecular therapeutic agents into the cytoplasm of mammalian cells remains a major barrier to their pharmaceutical applications. Cell-penetrating peptides (CPPs) are considered as potential carriers for cytoplasmic delivery of macromolecular drugs. However, due to the positive charge of most CPPs, strong non-specific cell membrane bindings may lead to relatively high toxicity. In this study, we report a series of anionic, CPP-mimicking, lysine-based hyperbranched polymers, which caused complete membrane disruption at late endosomal pH while remained non-lytic at physiological pH. The pH-responsive conformational alterations and the multivalency effect of the hyperbranched structures were demonstrated to effectively facilitate their interaction with cell membranes, thus leading to significantly enhanced membrane-lytic activity compared with their linear counterpart. The unique structures and pH-responsive cell-penetrating abilities make the novel hyperbranched polymers promising candidates for cytoplasmic delivery of biomacromolecular payloads.
1. INTRODUCTION

Intracellular delivery of biomacromolecular therapeutic agents including oligonucleotides, peptides and proteins is of critical importance for their efficacy\textsuperscript{1,2}. Compared with traditional small-molecule drugs, biomacromolecules are larger in size and cannot diffuse into cells. The translocation of biomacromolecules mostly relies on endocytosis\textsuperscript{3,4}. Following endocytic uptake, they are usually trapped in endosomes and susceptible to degradation by various enzymes in lysosomes eventually\textsuperscript{5}. Therefore, there is an increasing demand to design smart carriers to facilitate the escape of biomacromolecules from endosomes into the cytoplasm.

Cell-penetrating peptides (CPPs) are one of the widely used types of carriers for cytoplasmic delivery\textsuperscript{6–8}. The short peptides originally derived from natural proteins on the viral or bacterial surface, which are able to penetrate lipid membranes and translocate viruses or bacteria into the cell interior. Many CPPs are cationic, with a number of lysine or arginine residues, which are believed to play an important role in membrane penetration possibly due to their electrostatic interactions with negatively charged cell membranes\textsuperscript{9}. Many other CPPs are anionic, such as fusogenic peptides derived from the N-terminal sequence of the influenza virus hemagglutinin subunit HA2 and p28 derived from azurin\textsuperscript{10}, and their amphiphilic structure is believed to facilitate interactions with cell membranes, leading to subsequent successful membrane penetration\textsuperscript{11,12}. Unlike most cationic CPPs which induce direct membrane penetration at physiological pH, anionic amphiphilic CPPs cannot lyse cell membranes at physiological pH, but penetrate through endosomal membranes in the acidic environment. The pH-dependent endosomolytic activity of the anionic CPPs makes them more biocompatible than cationic ones, thus demonstrating promising potential for cytoplasmic drug delivery\textsuperscript{13}. 
Increasing interest has been focused on branched multivalent CPPs, which can have higher cell penetration, higher serum stability, reduced biodegradation and higher loading capacities than linear CPPs. Attention has thus far been mainly paid to cationic branched CPPs based on grafting of natural linear CPPs such as TAT to a hyperbranched or dendritic polymer backbone or synthesis of branched arginine-rich polypeptides. The results showed that a certain degree of branching should be achieved to enhance the membrane penetration efficiency of cationic CPPs, but could also unfavorably lead to an increased cytotoxicity.

Though many linear or branched CPPs have been reported to be able to deliver macromolecules or nanoparticles into mammalian cells, side effects including significant non-specific binding to cell membranes are potentially problematic. Also the costs for peptide production and purification are prohibitively high. Biomimetic amphiphilic polymers with similar membrane-penetrating properties are thus considered as less expensive and safer alternatives. Many efforts have been dedicated to developing guanidine-containing polymers which mimic arginine-containing CPPs. For example, guanidine-based methacrylamide copolymers, guanidinium-rich oligocarbonate and poly(disulfide) have been reported as effective molecule transporters. Despite the high efficiency, the cytotoxicity of those positively charged polymers remains a concern due to their strong ionic interaction with negatively charged cell membranes. The non-specific membrane bindings usually lead to high cytotoxicity and poor in vivo activity. By contrast, there have been few reports about non-cationic biomimetic membrane-penetrating polymers. Stenzel and his co-workers developed zwitterionic guanidine-based oligomers which were demonstrated to be much less toxic than their cationic counterparts.

A class of anionic, easy-to-synthesize, biodegradable polyamides has been recently developed to mimic pH-responsive, influenza viral fusogenic CPPs. The linear, metabolite-derived, parent
polymer, poly(l-lysine isophthalamide), only displayed the limited membrane-lytic capacity at lysosomal pH. Hydrophobic amino acids or alkyl chains were grafted onto its pendant carboxyl groups to manipulate its pH-responsive conformational change and endosomolytic activity\textsuperscript{27-31}, thus enabling efficient cytoplasmic delivery of small-molecule model drugs and bioactive macromolecules (e.g. therapeutic proteins and siRNA) \textit{in vitro} and \textit{in vivo}\textsuperscript{32-34}. Herein, we introduce a hyperbranched topology to increase the membrane penetrating activity of poly(l-lysine isophthalamide). The anionic, pH-responsive, lysine-based, hyperbranched polymers with different branching degrees were prepared by a facile one-pot synthetic strategy. To our knowledge, it is the first report of anionic, hyperbranched, CPP-mimicking polymers. As shown in Scheme 1, at physiological pH, the novel anionic polymers were relatively hydrophilic and had no membrane-penetrating ability. At late endosomal pH, they became more hydrophobic and displayed significantly increased membrane-lytic activity. The multivalency effect of their hyperbranched structures further enhanced their interaction with cell membranes. Efficient intracellular delivery and endosomal escape of calcine as a membrane-impermeable model drug indicates that the novel hyperbranched polymers are promising candidates for cytoplasmic delivery of therapeutic agents, in particular biomacromolecular drugs.

\textbf{Scheme 1}. Schematic showing the ability of the hyperbranched polymers to facilitate pH-responsive membrane penetration and endosomal escape.
2. MATERIALS AND METHODS

2.1. Materials

l-lysine methyl ester dihydrochloride was purchased from Alfa Aesar (Heysham, UK). Sodium phosphate dibasic, potassium chloride, potassium carbonate, sodium chloride, citric acid, sodium citrate, potassium phosphate monobasic, sodium hydroxide, ethanol, acetone, dimethyl sulfoxide (DMSO), hydrochloric acid, N-methyl-2-pyrrolidone (NMP), Texas Red® hydrazide, Hoechst 33342, Alamar Blue assay kits and LysoTracker® Red DND 99 were purchased from Thermo Fisher Scientific (Leicestershire, UK). Iso-phthaloyl chloride, 1,3,5-benzenetricarboxylic acid chloride, pyrene, calcein, fluorescein isothiocyanate–dextran (FITC-dextran, with various average molecular weights of 10K, 70K and 150K Da), deuterium oxide (D₂O), DMSO-d₆, melittin, Dulbecco’s modified Eagle’s medium (DMEM), fetal bovine serum (FBS), trypsin-EDTA and penicillin-streptomycin (Pen Strep) were purchased from Sigma-Aldrich (Dorset, UK). Sheep red blood cells (RBCs) were purchased from TCS Biosciences Ltd (Buckingham, UK). Pyrene was recrystallized by acetone and DMSO was dried by molecular sieves (4 Å) before use. Other chemicals and reagents were used directly as received.

2.2. Synthesis of Hyperbranched Poly (l-lysine iso-phthalamide) (HPLP)

l-lysine methyl ester dihydrochloride (0.01 mol) and potassium carbonate (0.04 mol) were dissolved in 50 mL pre-cooled deionized water. Iso-phthaloyl chloride (0.01 mol) and 1,3,5-benzenetricarboxylic acid chloride (0.1, 0.3, 0.5, 0.8 and 1 mmol respectively; i.e., 1, 3, 5, 8 and 10 mol% relative to l-lysine methyl ester dihydrochloride respectively) were dissolved in 50 mL pre-cooled dry acetone. The linear counterpart polymer poly (l-lysine iso-phthalamide) (PLP) was synthesized in the absence of 1,3,5-benzenetricarboxylic acid chloride and used as a
negative control for NMR analyses. The acetone solution was slowly added dropwise into the aqueous solution and the Teflon-coated magnetic stir bar was used to mix the mixture thoroughly. The reaction was allowed to proceed for 0.5 hour in an ice bath. The polymer precipitate was collected and washed with deionized water to remove excess solvent. After that, the polymer was left in an oven at 55 °C to dry overnight.

The dried powder of hyperbranched poly (L-lysine methyl ester iso-phthalamide) was dissolved in DMSO in a beaker. 5 wt% NaOH in anhydrous ethanol was added in excess to the polymer solution and the hydrolytic reaction was allowed for 0.5 hour at room temperature. The hydrolyzed polymer precipitate was collected by vacuum filtration and redissolved in deionized water. Then 1 M HCl aqueous solution was added to precipitate the polymer out. The precipitate was collected by vacuum filtration and redissolved in 1 M NaOH. The cycle was repeated three times and the final polymer solution was dialyzed against deionized water in the Visking membrane tubing with a molecular weight cut-off of 12K–14K Da (Medicell) for three days. After dialysis, the HPLP solution was acidified using 1 M HCl and the precipitate was collected and further dried by freeze-drying to obtain the acidic polymer for characterization.

The polymers in salt form were obtained from neutralization of the acidic polymers. A dilute NaOH solution (0.04 M) was used to fully dissolve the acidic polymer and the polymer solution was dialyzed overnight. After dialysis, the pH of HPLP solution was adjusted to around 7. The polymer solution was subsequently freeze-dried to achieve a white fine powder.

2.3. Polymer Structural Characterization

The acidic HPLPs were dissolved in a binary mixture of D₂O and DMSO-d₆ (1:2, volume ratio) and their NMR spectra were obtained on an Avance III 400MHz NMR spectrometer (Bruker,
Switzerland) at room temperature. The integrals of the peaks (7.95-8.45 ppm) of the HPLPs and their linear counterpart PLP were obtained, using a typical peak of the linear polymer without crosslinker (7.25-7.48 ppm) as reference, to calculate the crosslinker percentage (Equation 1).

\[
\text{Crosslinkers (\%)} = \frac{\text{peaks (7.95-8.45 ppm) of HPLPs} - \text{peaks (7.25-8.45 ppm) of linear PLP}}{3} \times 100\%
\]  

(1)

Gel permeation chromatography (GPC) analyses of the polymers in acidic form were performed using NMP as the mobile phase and a 300-mm long, 7.5-mm (internal diameter) wide, polystyrene/polydivinylbenzene-packed, Mixed-D column (Polymer Laboratories, UK). Detection was made by UV absorbance using a Knauer diode array Smartline 2600 detector at 270, 300, 350, and 370 nm respectively. The system was operated at 80 °C and a constant flow rate of 0.5 mL min⁻¹. Polystyrene standards were used for calibration.

The FTIR spectra of the acidic HPLPs were obtained on a Spectrum 100 Fourier transform infrared instrument (Perkin Elmer, USA).

Differential scanning calorimetry (DSC) analyses of the HPLPs were evaluated using a TA Q200 differential scanning calorimeter (Perkin Elmer, USA). Typically, a specific acidic HPLP was filled on a platinum plate, which was heated to 200 °C and then cooled to 0 °C in one thermal cycle. The thermal cycle was repeated twice and the ramp rate was 10 °C per minute.

2.4. Turbidity Test

The HPLP polymers in salt form were dissolved in phosphate buffer at pH 7.4 by sonication, at a concentration of 10 mg mL⁻¹. The stock solutions were diluted ten times with citric buffer solutions at different pHs (from 3.6 to 5.4) and left for 48 hours before measurement. The
transmittance of polymer solution was measured at 480 nm using a GENESYS 10S UV/Vis spectrophotometer (Thermo Fisher Scientific, USA).

2.5. Fluorescence Spectroscopy

Pyrene was used to investigate the pH-responsive changes of polymer amphiphilicity. 1 mM pyrene stock solution in methanol was prepared and stored in dark. This pyrene stock solution was diluted with citric buffers at different pHs (from 3.6 to 5.4) to make a final concentration of pyrene at 600 nM. The specific polymer stock solution was diluted to certain concentrations by the pyrene-containing citric buffer solutions. Each sample was wrapped with foil and left in dark for 24 hours before fluorescence measurement. The excitation spectra ($\lambda_{em} = 390$ nm) were obtained using a Jobin Yvon Horiba FluoroMax-4 spectrofluorometer (HORIBA, UK) at right-angle geometry. The width of the bandpass filter for emission was 1 nm and that for excitation was 5 nm. The ratio of pyrene fluorescence intensities at 338 to 333 nm ($I_{338}/I_{333}$) in the excitation spectra was used to examine the polarity of pyrene environment.

The critical aggregation concentration (CAC) was measured using the same method. The specific polymer stock solution was diluted in pH 7.4 phosphate buffer containing 600 nM pyrene to reach a series of final polymer concentrations (from 10 ng mL$^{-1}$ to 5 mg mL$^{-1}$). Each sample was wrapped with foil and left in dark for 24 hours before fluorescence measurement. $I_{338}/I_{333}$ in the excitation spectra was recorded under the same setting described above.

2.6. Dynamic Light Scattering (DLS)

The HPLP polymers in the pH 4.6 citrate buffer and the pH 7.4 phosphate buffer at a concentration of 1 mg mL$^{-1}$ were used for DLS analysis. Before DLS measurement, 0.45 µm
cellulose filters were used to filter large particles out of the polymer sample solutions. The dynamic light scattering was measured at an angle of 90° in a 10-mm diameter cell at 25 °C (Zetasizer Nano S, Malvern, UK). Eleven scans were run for each measurement, which was repeated in triplicate. The particle size and the polydispersity index (PDI) were calculated by Malvern software.

2.7. Transmission Electron Microscopy (TEM)

The HPLP solution (1 mg mL$^{-1}$) was dropped on a 200-mesh copper grid coated with a holey carbon film. Excess amount of solution was removed by filter paper. The grid was dried in the air overnight before TEM measurement. Images were captured by a JEOL 2000FX TEM (USA).

2.8. Hemolysis Assay

The membrane-lytic activities of the HPLPs were examined using the previously established hemolysis assay$^{30}$. Briefly, sheep RBCs were washed with 150 mM NaCl three times before use. Then RBCs were resuspended in polymer buffer solutions at different pHs (from 4.5 to 7.4) to achieve a final RBC density within the range of 1-2 × 10$^8$ cell mL$^{-1}$. For the hemolysis of melittin, RBCs were resuspended in 150 mM NaCl at different pHs. RBCs resuspensions without polymers or melittin served as negative controls while RBCs in deionized water as the positive control. Both samples and controls were placed in a shaking water bath at 37 °C for an hour, followed by centrifugation at 4000 rpm for 4 min. The absorbance of the supernatants was measured at 540 nm by a GENESYS 10S UV/Vis spectrophotometer (Thermo Fisher Scientific, USA) and relative hemolysis was calculated. Three replicates were used for each sample and results were presented as mean ± standard deviation (SD).
2.9. Confocal Microscopy Imaging of RBCs

After washing with 150 mM NaCl three times, sheep RBCs were resuspended in the pH 5.0 citrate buffer (100 mM) at a density of 2 ×10^7 cell mL\(^{-1}\). To the RBC suspensions were added FITC-dextran with different molecular weights (10K, 70K and 150K Da, final concentration at 10 µM) and Texas Red® hydrazide (final concentration at 1.5 µM), followed by the addition of HPLP5 (final concentration at 1 mg mL\(^{-1}\)). Controls were RBCs in buffers without HPLP5. Images were recorded using an LSM-510 inverted laser scanning confocal microscope (Zesis, Germany) at the excitation wavelengths of 488 nm for FITC-dextran and 543 nm for Texas Red® hydrazide.

2.10. Cell Culture

HeLa adherent epithelial cells (human cervical cells) were grown in DMEM containing 10% (v/v) FBS, 100 units mL\(^{-1}\) penicillin and 100 µg mL\(^{-1}\) streptomycin unless specified. The HeLa cells were trypsinized using trypsin-EDTA and maintained in a humidified incubator with 5% CO\(_2\) at 37°C.

2.11. Alamar Blue Cytotoxicity Assay

The in-vitro cytotoxicity of the HPLPs was determined by Alamar Blue assay. Briefly, HeLa cells were first seeded in a 96-well microplate at a density of 7×10^4 cell mL\(^{-1}\) and cultured for 24 hours. After that, the cells were then treated with 0.22 µm filter-sterilized HPLP solutions in DMEM (0.01 – 5 mg mL\(^{-1}\)) or melittin solutions in DMEM (0.1 – 50 µg mL\(^{-1}\)), while the cells without exposure to the polymers or melittin were used as control. After 24 hours of treatment, the polymer-containing medium was removed, followed by the addition of the complete DMEM
containing 10% (v/v) alamar blue. The plate was further incubated for 4 hours before fluorescence measurement using a GloMax®-Multi+ Microplate Multimode Reader (Promega, USA) at the excitation wavelength of 525 nm and the emission wavelength of 580-640 nm. The wells with alamar blue but without cells served as background. Cell viabilities were calculated from the fluorescent readings. Five replicates were used for each sample and results were presented as mean ± SD.

2.12. Cytoplasmic Delivery of Endocytosed Materials

Calcein (membrane-impermeable model drug) and HPLP5 were dissolved in serum-free DMEM and filtrated by a 0.22-µm filter before use. HeLa cells were seeded in a 35-mm glass-bottom petri dish (MatTek, USA) at a density of 5×10⁵ cell mL⁻¹ and cultured for 24 hours. The spent medium was removed and replaced with 2 mL of the serum-free DMEM containing 2 mg mL⁻¹ calcein and 1 mg mL⁻¹ HPLP5. The cells treated with calcein in the absence of HPLP5 were used as control. After 1 hour of treatment, the calcein-containing DMEM was removed. The cells were washed three times with PBS buffer and replenished with complete DMEM for a further incubation of 5 hours. Before imaging, the cells were treated with Hoechst 33342 and LysoTracker® for 20 minutes in the incubator, and then washed three times with PBS buffer. The cells were imaged using a Zesis LSM-510 inverted laser scanning confocal microscope at the excitation wavelengths of 405 nm for Hoechst 33342, 488 nm for calcein and 543 nm for LysoTracker®.

3. RESULTS AND DISCUSSION

3.1. Synthesis and Structural Characterization of HPLPs
As illustrated in Scheme 2, HPLP precursors (e.g. methyl ester forms of the HPLPs) were synthesized by a facile one-pot polycondensation reaction. An amino acid derivative, L-lysine methyl ester dihydrochloride, was used as a di-amine building block and *iso*-phthaloyl chloride as a di-chloride building block, while 1,3,5-benzenetricarboxylic acid chloride was introduced as a crosslinker to achieve hyperbranched structures. Different ratios of the crosslinker relative to L-lysine methyl ester dihydrochloride (1, 3, 5, 8 and 10 mol%) were employed, leading to the polymers with different branching degrees. The HPLPs were finally obtained after a hydrolysis of the precursor polymers to reveal carboxylic acid groups.

Scheme 2. Outline for the synthesis of HPLP polymers.

The structures of the HPLP polymers with different branching degrees (i.e., HPLP1, HPLP2, HPLP3, HPLP4, and HPLP5) were characterized by $^1$H NMR. Due to the same chemical composition, all the HPLPs had similar peaks (Figure S1). Figure 1a shows a typical spectrum of a hyperbranched polymer dissolved in a binary mixture of D$_2$O and DMSO-d$_6$ (1:2, v/v), which was utilized to suppress the peaks of amide groups thus revealing the peaks of the branching point at 8.2-8.4 ppm (zoomed-in spectra shown in Figure 1b). It is obvious that the peaks of the branching point in HPLP5, which incorporated the highest amount of crosslinker, were much
higher than those in HPLP1 with the lowest amount of crosslinker. Thus, the crosslinker percentage could be calculated from the integration of these peaks (see Equation 1 in the Section 2.3) and the calculated results are shown in Table 1.

![Figure 1](image_url)

**Figure 1.** (a) $^1$H NMR spectrum of HPLP5 with proton designations. (b) Zoomed-in $^1$H NMR spectra of HPLP1, HPLP3 and HPLP5 between 6.9 and 9.1 ppm. The solvent used was a binary mixture of D$_2$O and DMSO-d$_6$ (1:2, v/v).

**Table 1.** Characteristics of HPLP polymers with different branching degrees

<table>
<thead>
<tr>
<th>Polymer</th>
<th>Theoretical crosslinker percentage</th>
<th>Experimental crosslinker percentage</th>
<th>$M_n \times 10^6$</th>
<th>$M_w \times 10^6$</th>
<th>PDI</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPLP1</td>
<td>1%</td>
<td>3%</td>
<td>1.10</td>
<td>1.87</td>
<td>1.70</td>
</tr>
<tr>
<td>HPLP2</td>
<td>3%</td>
<td>4%</td>
<td>0.99</td>
<td>1.99</td>
<td>2.01</td>
</tr>
<tr>
<td>HPLP3</td>
<td>5%</td>
<td>7%</td>
<td>1.06</td>
<td>2.71</td>
<td>2.56</td>
</tr>
<tr>
<td>HPLP4</td>
<td>8%</td>
<td>9%</td>
<td>1.64</td>
<td>4.34</td>
<td>2.65</td>
</tr>
<tr>
<td>HPLP5$^d$</td>
<td>10%</td>
<td>12%</td>
<td>1.99</td>
<td>4.67</td>
<td>2.34</td>
</tr>
</tbody>
</table>
a Determined by the feeding ratio of crosslinker in the polycondensation reaction. \(^\text{b}\) Determined by \(^1\)H NMR. \(^\text{c}\) Determined by GPC. \(^\text{d}\) HPLP5 was not dissolved well in NMP as the GPC mobile phase.

As shown in Table 1, the calculated percentages of the crosslinker exhibited a similar trend with the theoretical data determined by the stoichiometric ratios employed in the polymerization although the corresponding experimental values were a bit higher, which further proved that the hyperbranched polymers with different crosslinking degrees were successfully prepared. It also indicates that the crosslinking degrees could be readily adjusted by varying the feeding ratio of crosslinker.

The hyperbranched structure of HPLPs was further consolidated by GPC (Table 1). The average molecular weight, both number average molar mass (\(M_n\)) and mass average molar mass (\(M_w\)), generally increased from HPLP1 to HPLP5. The PDI was increased from 1.70 for HPLP1 to 2.65 for HPLP4. However, HPLP5 showed a lower PDI value (2.34) than HPLP4, possibly due to the reduced solubility in the GPC solvent for HPLP5 with a sufficiently high branching degree and as a result the filtration of undissolved species with higher molecular weights and branching degrees before GPC measurement.

FTIR spectra in Figure S2 show the typical amide I and II bands at 1623 and 1527 cm\(^{-1}\) respectively. However, the spectra of all the HPLPs were similar because of the high similarity of their polymer backbones. DSC results in Figure S3 revealed an increased glass transition temperature \(T_g\) for the HPLP polymer with a higher branching degree, from 150.6 °C for HPLP1 to 172.3 °C for HPLP5. This is in agreement with the previous results of thermal analyses of
amorphous polymers. As the branching degree increased, the polymer architecture became more compact due to the introduction of more junction points. The compact structure hindered the free movement of polymer chains, resulting in an increased $T_g$.

### 3.2. pH-Responsive Conformational Alterations

To examine their pH-responsive physiochemical properties, the transmittance of the HPLP solutions in different pH buffers was recorded. As shown in Figure 2a, HPLP1 with the lowest branching degree showed a sharp transition from pH 4.6 to 4.3, while a broader transition from pH 5.0 to 4.2 was observed for HPLP5 with the highest branching degree. This broadening of the pH range for phase transition might be due to the increased level of confinement and the consequent gradual conformational change with increasing the branching degree.

The pH-induced alteration of HPLP hydrophobicity was examined by spectrofluorimetry using pyrene as a microenvironmental-polarity probe. Both emission and excitation spectra of pyrene can be significantly changed when it migrates from polar to non-polar environments. Herein, the ratio of intensities at 338 to 333 nm ($I_{338}/I_{333}$) in the excitation spectra was used to estimate the polarity of pyrene environment. A high $I_{338}/I_{333}$ value indicates that pyrene was solubilized in a relatively more hydrophobic microenvironment, and vice versa.
**Figure 2.** (a) pH-dependent transmittance of HPLP1, HPLP3 and HPLP5 at 1 mg mL\(^{-1}\) in 100 mM buffers. (b) Variations in \(I_{338}/I_{333}\) of HPLP1, HPLP3 and HPLP5 at 0.1 mg mL\(^{-1}\). (c) Variations in \(I_{338}/I_{333}\) of HPLP1, HPLP3 and HPLP5 at 2 mg mL\(^{-1}\). (d) CAC determination for HPLP1 and HPLP5 at pH 7.4.

Figure 2b illustrates the variations in \(I_{338}/I_{333}\) ratios as a function of pH in the excitation spectra of pyrene dissolved in 0.1 mg mL\(^{-1}\) HPLP aqueous solutions. The hyperbranched polymer with a higher branching degree showed a less steep transition. At pH 5.4, the \(I_{338}/I_{333}\) ratios of pyrene dissolved in the solutions of all the three HPLPs tested were close to that in deionized water (~0.6)\(^{38}\), which suggests that pyrene was in a hydrophilic microenvironment. As pH decreased to
4.0, the $I_{338}/I_{333}$ ratio increased to 1.0, 0.9 and 0.8 for HPLP1, HPLP3 and HPLP5 respectively. This indicates that all the three polymers displayed conformational changes and as a result hydrophobic domains were formed, with the polymer at a higher branching degree forming less hydrophobic domains at low pH. This could be attributed to the stronger steric hindrance within a more branched polymer, which could hinder the pH-induced conformational change and hydrophobic domain formation.

At a higher polymer concentration of 2 mg mL$^{-1}$, the $I_{338}/I_{333}$ showed a similar trend but higher values (Figure 2c). At pH 5.4, the $I_{338}/I_{333}$ ratios of HPLP5 and HPLP3 were around 0.9 and 0.85 respectively, indicative of the formation of some hydrophobic domains. Considering their amphiphilic nature, the polymers could form multimolecular aggregates even at neutral pH, thus bearing hydrophobic cores which accommodated pyrene. To confirm this hypothesis, the critical aggregation concentration (CAC) was determined using pyrene to probe the hydrophobicity variations. As shown in Figure 2d, HPLP1 and HPLP5 had a CAC of 1 and 0.3 mg mL$^{-1}$ respectively. The pH-responsive conformational alterations of the hyperbranched polymers were different below and above their CAC. As shown in Figure 2b, when the polymer concentration (0.1 mg mL$^{-1}$) was below the CAC, the pH-sensitive conformational changes predominantly resulted from intramolecular interactions. However, when the polymer concentration was above the CAC, the HPLPs tended to form aggregates due to intermolecular interactions. As shown in Figure 2c, at a polymer concentration (2 mg mL$^{-1}$) sufficiently higher than its CAC (0.3 mg mL$^{-1}$), most HPLP5 molecules in the solution could form multimolecular aggregates, wherein their hydrophobic cores were independent of pH, while their hydrophilic shells remained pH-responsive. As pH decreased, the carboxyl groups present in the hydrophilic shells were protonated, enabling the shells to become hydrophobic gradually and the consequent formation
of the aggregates with more compact structures. In contrast, with a higher CAC (1 mg mL$^{-1}$) a significant amount of HPLP1 molecules could still display a distinct transition of conformation upon pH reduction at 2 mg mL$^{-1}$.

The pH- and concentration-dependent conformational alterations were further confirmed by DLS and TEM (Figure 3). At a concentration of 1 mg mL$^{-1}$, the average hydrodynamic size in diameter of HPLP1 was only 5.7 ± 1.6 nm at pH 7.4, which suggests that the hyperbranched polymer might form unimolecular micelles. At lower pH (4.6), HPLP1 aggregated into hydrophobic assemblies, with an increased average size of 25.3 ± 2.2 nm. In comparison, the mean size of HPLP5 at pH 7.4 was 256.7 ± 31.4 nm, confirming the formation of large multimolecular aggregates at the concentration sufficiently higher than its CAC as suggested in Figure 2. As pH dropped to 4.6, the shells of multimolecular aggregates became hydrophobic due to protonation of carboxyl groups and the whole aggregates shrank into more compact assemblies. A similar size change in response to pH has been previously reported for the linear PLP$^{39}$. HPLP5 aggregated into smaller particles (15.0 ± 2.0 nm) at the acidic pH than HPLP1 (25.3 ± 2.2 nm), possibly because the former polymer with a higher branching degree formed a more compact structure. The typical TEM images of HPLP5 at pH 4.6 and pH 7.4 were presented in Figure 3c and 3d respectively to validate its size decrease upon pH reduction as determined by DLS (Figure 3b). Small and dark dots around 15 nm in Figure 3c were considered as hydrophobic compact assemblies, while amorphous and diffused particles around 150 nm in Figure 3d were probably multimolecular aggregates. No obvious core-shell structure was observed, possibly due to the random assembly of the hyperbranched polymers.

Tian et al. reported the aggregation behaviour of the amphiphilic hyperbranched polymer based on poly(N-vinylcaprolactam) and poly(N,N-diethyl aminoethyl methacrylate) in aqueous
solution\textsuperscript{40}. Below its CAC, the polymer formed unimolecular micelles; while above its CAC it formed multimolecular micelles. The multimolecular micelles consisted of hydrophobic cores that could accommodate hydrophobic payloads and hydrophilic shells that could keep hydrated. This is consistent with our amphiphilic HPLP system at neutral pH. As pH decreased, the HPLPs in both unimolecular and multimolecular states became hydrophobic and as a result formed compact particles. As the CAC of polymer is structure-dependent, the HPLP with a higher branching degree would have a lower CAC due to the multivalency effect (Figure 2d). Therefore the pH-responsive conformational alterations of the HPLPs could be adjusted by varying hyperbranching degrees.
Figure 3. Hydrodynamic particle size distributions of (a) HPLP1 and (b) HPLP5 at 1 mg mL\(^{-1}\) in buffers at pH 4.6 (red) and pH 7.4 (black). TEM images of HPLP5 at (c) pH 4.6 and (d) pH 7.4 (scale bar 200 nm) with one representative particle shown in their respective insets (scale bar 50 nm).

3.3. pH-Responsive Membrane-Lytic Activity

The interaction between the HPLPs and cell membranes was investigated using sheep RBCs as a model. The RBCs were selected as a simple model system because they have similar membrane compositions and cytoskeleton structure as other mammalian cells, but no nucleus or other
organelles. Figure 4a shows the concentration-dependent hemolysis results of HPLP1, HPLP2, HPLP3, HPLP4 and HPLP5 at pH 5.0 characteristic of late endosomes. At a concentration lower than 0.5 mg mL\(^{-1}\), all the five HPLPs showed relatively low membrane-lytic activity, with the degrees of hemolysis below 30%. As the concentration increased, the HPLP with a higher branching degree showed a higher level of hemolysis. Almost 100% hemolysis was achieved by HPLP5 at 2 mg mL\(^{-1}\). The significantly enhanced membrane-lytic activity with increasing the branching degree was probably due to the multivalency effect of hyperbranched polymers. Other researchers have extensively reported the binding of dendritic polymers with bio-targets. For instance, Haynes et al. reported that hyperbranched polyglycerol-based mannose conjugates exhibited the much stronger interaction with the target lectin than their linear counterparts\(^1\). Furthermore, iron oxide nanoparticles functionalized with dendritic displays of mannose showed 1-2 orders of magnitude higher binding affinity with the protein Concanavalin A than those coated with nondendritic systems\(^2\). Herein, when the HPLPs interact with cell membranes, their globular structure and high density of surface groups facilitated their insertion into the lipid membranes, leading to the considerably higher binding affinity and membrane-lytic activity. Though the interaction with lipid membranes is more complicated than the binding with bio-targets, multivalent interactions could still contribute to the molecular recognitions, and thus facilitate the membrane interactions.

Figure 4b shows the pH-dependent membrane-disruptive activities. All the five HPLPs were efficient in membrane disruption at late endosomal pH (5.0), but essentially non membrane-lytic at physiological pH (7.4). At pH 7.4, HPLPs were deprotonated and relatively hydrophilic. The negatively charged cell membranes were less likely to interact with the anionic polymers due to the electronic repulsion. However, at pH 5.0 the protonation of carboxylic acid groups in the
HPLPs led to the formation of hydrophobic domains, which as a result triggered the significantly enhanced membrane interaction and subsequent considerable hemolysis. When pH decreased to 4.5, most polymers precipitated out of the aqueous solution, thus showing almost no membrane disruption. Figure S4 shows the hemolytic activity of melittin as a positive control, which is a commercially available, highly membrane-lytic CPP derived from bee venom. At 5 µg mL⁻¹, melittin induced almost complete hemolysis within the whole pH range (4.5 – 7.4) tested. The high membrane-lytic activity of CPPs at physiological pH is unfavorable for drug delivery due to their potential significant side effects. In contrast, HPLPs showed efficient hemolysis within the narrow late endosomal pH range, which made it possible to have a precise control of membrane-lytic activity; therefore the cytotoxic effects would be minimized and the controlled release of payloads could be achieved in a specific step of the whole endocytosis pathway.

Figure 4. (a) Concentration-dependent relative hemolysis of sheep RBCs in the presence of different HPLPs at pH 5.0. (b) pH-dependent relative hemolysis of sheep RBCs in the presence of different HPLPs at 2 mg mL⁻¹.
The mechanism of hemolysis was investigated by confocal microscopy. As shown in Figure 5, hemoglobin release was accompanied by the formation of ghost RBCs, which were visible in the bright field and could be more clearly visualized through staining with Texas Red® hydrazide. Those ghost cells, though permeable to hemoglobin, remained integrated at a microscopic scale without membrane collapse or fusion. Therefore, the mechanism of hemolysis mediated by the HPLPs could be pore formation in the RBC membranes, instead of membrane solubilization. By treating sheep RBCs with 10 µM FITC-dextran of different molecular weights and 1 mg mL⁻¹ HPLP5 at pH 5.0, it was found that FITC-dextran 10K could efficiently diffuse into the resulted ghost cells, appearing as the homogeneous intracellular distribution of bright green fluorescence (Figure 5a). However, FITC-dextran 70K with a Stokes’ radii of approximately 6 nm⁴⁴ (Figure 5b) and FITC-dextran 150K (Figure 5c) were partially inside the ghost cells, as the fluorescence intensity inside the ghost cells was higher than inside the intact cells but lower than the background. This indicates that molecules below a size threshold around 6 nm in diameter could diffuse freely across the ghost cell membranes, while molecules larger than the threshold could only partially permeate through the membranes. This was validated by the simultaneous diffusion curves of FTIC-dextran 10K and Texas Red® hydrazide (Figure S5). In comparison, there was no formation of ghost cell and no intracellular loading of FITC-dextran when the RBCs were treated with FITC-dextran 10K at pH 5.0 but without the HPLP polymer (Figure 5d), suggesting that the formation of ghost cells and the cross-membrane transport were induced by the HPLP-membrane interaction. Furthermore, no ghost cells were observed when the RBCs were treated with FITC-dextran and the hyperbranched polymers at pH 4.5 and 7.4 (Figure S6), which was consistent with the hemolysis results shown in Figure 4b.
Figure 5. Confocal microscopy images of sheep RBCs treated with 10 µM different sized FITC-dextran and 1.5 µM Texas Red® hydrazide at pH 5.0 in the presence or absence of 1 mg mL⁻¹ HPLP5: (a) FITC-dextran 10K and HPLP5; (b) FITC-dextran 70K and HPLP5; (c) FITC-dextran 150K and HPLP5; (d) FITC-dextran 10K in the absence of HPLP5. Scale bar 10 µm.

In addition, time-lapse confocal microscopy analysis was performed to reveal the morphology changes of erythrocytes prior to hemolysis (see Movie S1 and Figure S7). Treated with HPLP5 at 1 mg mL⁻¹ at pH 5.0, most RBCs rearranged their membranes vigorously. Originally most RBCs were discocytes. Then a great number of stomatocytes were observed before evolving into
spherocytes. Eventually those swollen spherical RBCs became ghosts. Similar morphology variations were reported by other researchers on the erythrocytes treated with CPPs, e.g., snake venoms\(^45\) and the HA2-TAT peptide combining the HA2 peptide from influenza virus and the TAT peptide from HIV\(^46\). As shown in Figure S6, in the stage when stomatocytes were formed, minor membrane defects might already exist in the RBC membranes, as evidenced by the transport of some FITC-dextran 10K molecules into the cell interior. But the membrane alteration was not sufficient for hemoglobin leakage. When stomatocytes swelled and became spherocytes, the defects in the RBC membranes were exaggerated and finally allowed the hemoglobin release without cell lysis. This led to the formation of ghost RBCs and the consequent diffusion of a significant amount of FITC-dextran 10K into the ghost cells, reaching the same green fluorescence intensity between the exterior and interior.

### 3.4. Evaluation of Cytotoxicity and Cytoplasmic delivery

The effect of the HPLPs toward the metabolic activity of HeLa cells was evaluated by Alamar Blue assay. As shown in Figure 6, all the five HPLPs had no cytotoxic effect within the entire concentration range (0.01–5 mg mL\(^{-1}\)) tested, indicating that the lysine-based hyperbranched polymers were quite biocompatible. As comparison, the positive control CPP melittin showed significant cytotoxicity against HeLa cells (with the relative viability below 13%) at the concentration \(\geq 0.01\) mg mL\(^{-1}\) (Figure S8).
Figure 6. Concentration-dependent relative viabilities of HeLa cells treated with different HPLPs for 24 hours as determined by Alamar Blue assay.

Membrane-impermeable calcein was used as a tracer molecule to investigate whether the HPLPs could facilitate the release of endocytosed model drugs from intracellular vesicles into the cytoplasm of mammalian cells. Specifically, HeLa cells were co-incubated with both 1 mg mL$^{-1}$ HPLP5 and 2 mg mL$^{-1}$ calcein. After 1 hour of internalization at 37 °C, the cells were further incubated in fresh complete DMEM for 5 hours before imaging, to allow maturation of endosomes and transformation of the polymer to a membrane-lytic state upon acidification. As shown in Figure 6a, when the cells were treated with calcein only, the membrane-impermeable fluorophore appeared as green spotty dots, which were mainly colocalized with red LysoTracker. This indicates that the endocytosed calcein remained trapped in endosome/lysosomes in the absence of HPLP5. In contrast, uptake of HPLP5 led to strong diffuse green fluorescence.
throughout the cell interior (Figure 6b). This suggests that upon protonation of HPLP5 in late endosome/lysosome, the hyperbranched polymer destabilized the membrane of intracellular vesicles and released calcein into the cell cytoplasm efficiently.

![Figure 7](image)

**Figure 7.** Confocal microscopy images of HeLa cells showing the intracellular distribution of the endocytosed calcein. The cells were treated with (a) 2 mg mL\(^{-1}\) calcein only, and (b) both 2 mg mL\(^{-1}\) calcein and 1 mg mL\(^{-1}\) HPLP5. Images were collected at 5 hours after 1 hour of uptake. Scale bar 20 µm.

### 4. CONCLUSIONS

Five HPLPs with various branching degrees have been synthesized and their structures have been characterized. The NMR and GPC data demonstrated that the branching degrees increased with increasing the feeding ratios of crosslinkers. Turbidimetry, fluorescence spectroscopy, DLS and TEM measurements demonstrated that all the HPLPs displayed pH-induced conformational
changes. Unimolecular micelles or multimolecular aggregates with hydrophobic cores were formed at neutral pH depending on if the polymer concentration was below or above the CAC, and both could become hydrophobic particles at acidic pH. Since the CAC was dependent on the branching degree, the pH-responsive conformational change could be manipulated by simply controlling the hyperbranched structure. The HPLPs displayed pH-responsive hemolytic activity. At neutral pH, they were necessarily non-haemolytic; while at late endosomal pH, they could hemolyze almost 100% erythrocytes. Molecules smaller than 6 nm could diffuse into the resulted ghost cells, which indicated that the mechanism of hemolysis could be pore formation instead of membrane solubilization. All the HPLPs showed negligible cytotoxicity toward HeLa cells at a concentration as high as 5 mg mL\(^{-1}\), but could facilitate the endocytosed payload to efficiently escape from intracellular vesicles into the cell cytoplasm. Compared with traditional CPP-mimicking polymers, the novel, biocompatible, anionic HPLPs with unique lysine-based hyperbranched structures demonstrated pH-induced considerable membrane destabilization, suggesting their potential applications in efficient cytoplasmic drug delivery.

ASSOCIATED CONTENT

SUPPORTING INFORMATION

The Supporting Information is available free of charge on the ACS Publications website at http://pubs.acs.org. Additional structural characterization data including NMR, FTIR and DSC; hemolytic activity of melittin; diffusion kinetics of FTIC-dextran and Texas Red\(^{\circledR}\) hydrazide into ghost RBCs; confocal microscopy images of sheep RBCs treated with HPLP5, FITC-dextran and Texas Red\(^{\circledR}\) hydrazide at pH 4.5 and 7.4; time-lapse confocal microscopy images of RBCs
treated with HPLP5, FITC-dextran and Texas Red® hydrazide; relative viabilities of HeLa cells treated with melittin; and the movie (AVI) showing the formation of HPLP5-induced ghost RBCs.

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Notes

The authors declare no competing financial interests.

DATA ACCESS STATEMENT

Data underlying this article can be accessed on Zenodo (www.zenodo.org) at DOI: 10.5281/zenodo.192495, and is openly available under the Creative Commons CC-BY licence.

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