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Membrane-Anchoring, Comb-Like Pseudopeptides for Efficient, pH-Mediated Membrane Destabilization and Intracellular Delivery

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KEYWORDS: pH-responsive; pseudopeptide; endosomal escape; drug delivery; comb-like polymer

ABSTRACT

Endosomal release has been identified as a rate-limiting step for intracellular delivery of therapeutic agents, in particular macromolecular drugs. Herein, we report a series of synthetic pH-responsive, membrane-anchoring polymers exhibiting dramatic endosomolytic activity for efficient intracellular delivery. The comb-like pseudopeptidic polymers were synthesized by grafting different amounts of decylamine (NDA), which act as hydrophobic membrane anchors, onto the pendant carboxylic acid groups of a pseudopeptide, poly (L-lysine *iso*-phthalamide). The effects of the hydrophobic relatively long alkyl side chains on aqueous solution properties, cell membrane destabilization activity and *in-vitro* cytotoxicity were investigated. The optimal polymer containing 18 mol% NDA exhibited limited hemolysis at pH 7.4, but induced nearly complete membrane destabilization at endosomal pH within only 20 min. The mechanistic investigation of membrane destabilization suggests the polymer-mediated pore formation. It has been demonstrated that the polymer with hydrophobic side chains displayed a considerable endosomolytic ability to release endocytosed materials into the cytoplasm of various cell lines, which is of critical importance for intracellular drug delivery applications.

1. INTRODUCTION

Efficient cytoplasmic delivery of therapeutic agents, in particular macromolecular drugs, remains a key limitation to their therapeutic effect.¹ Without egress, the endocytosed contents are generally routed for lysosomal degradation.² Influenza viruses can utilize hemagglutinin HA-2 N-terminal fusogenic peptides to disrupt the endosomal membrane at acidic pH and allow efficient escape of the viral nucleic acid from the endosome into the cytoplasm before the lysosomal fusion process occurs.^{3,4} However, the clinical application of viral vectors for drug delivery is usually limited due to safety issues and difficulties in large-scale production. It is therefore increasingly important to develop endosomolytic non-viral vectors, which can release the internalized active compounds into the cytoplasm without lysosomal degradation.^{5–8}

Particular interest has been focused on development of anionic polymers as drug carriers to mimic the pH-dependent membrane disruptive behavior of fusogenic viral peptides.^{9–11} The polyanions containing both hydrophobic segments and weakly ionizable carboxyl groups can exhibit more favorable biodistribution and significantly lower cytotoxicity compared to polycations.¹² As pH decreases below their pKa, they display a coil-to-globule conformational change due to the protonation of carboxylate ions, which, as a result, leads to lipid membrane destabilization.¹³ Manipulating the hydrophobic/hydrophilic balance of the anionic polymers is crucial to enhance their membrane destabilization activity.^{14–16} Efforts on improving hydrophobicity of the anionic endosomolytic polymers have been so far mainly focused on incorporation with short hydrophobic pendant groups, such as vinyl poly(α -alkylacrylic acid) polymers bearing pendant C1–C5 alkyl groups^{17,18} and pseudopeptidic polymers bearing pendant hydrophobic natural amino acids¹⁹.

In nature, however, many membrane proteins contain covalently attached long-chain fatty acids, which can act as hydrophobic anchors inserting into the lipid bilayer to facilitate the

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3 membrane binding. These membrane proteins may have more than one attached long-chain
4 moiety to ensure sufficiently strong hydrophobic interaction with the membrane.²⁰ Inspired
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6 by the membrane-anchoring proteins, long aliphatic chains can be incorporated onto a
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8 polymer backbone to form a “comb-like” polymer architecture, which is distinct from the
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10 linear or branched polymer, to enhance the polymer-membrane interaction.
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14 Eccleston *et al.* have developed a biodegradable, metabolite-derived, anionic pseudopeptide,
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16 poly(L-lysine *iso*-phthalamide) (PLP) consisting of a hydrophobic backbone and pendant
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18 carboxyl groups.²¹ The amphiphilic pseudopeptide does not contain long aliphatic chains that
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20 are present in many membrane proteins, and displays low cell membrane disruptive capacity
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22 only at relatively low pH (4.5–5.0), which is outside the endosomal pH range.²² Herein, we
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24 report a series of comb-like polymers synthesized by grafting hydrophobic decylamine (NDA)
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26 onto the carboxylic acid groups along the PLP backbone at different densities. The effects of
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28 the conjugated relatively long chains on the pH-responsiveness of polymer conformation and
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30 membrane destabilization were investigated. The mechanism of cell membrane disruption
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32 mediated by the comb-like polymers was elucidated. Their cytotoxicity and ability to release
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34 endocytosed materials from endosomes into the cytoplasm of different types of cancerous and
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36 non-cancerous mammalian cells were also evaluated.
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43 2. MATERIALS AND METHODS

44 2.1. Materials

45
46 Decylamine, pyrene, calcein, fluorescein isothiocyanate–dextran (FITC-dextran, average
47
48 M_w of 4k, 10k, 70k, 150k and 2000k Da respectively), *iso*-phthaloyl chloride, Dulbecco's
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50 modified Eagle's medium (DMEM), fetal bovine serum (FBS), Eagle's minimum essential
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52 medium (MEM) non-essential amino acid solution, penicillin and Dulbecco's phosphate
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54 buffered saline (D-PBS) were purchased from Sigma Aldrich (Dorset, UK). Dimethyl
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3 sulfoxide (DMSO), triethylamine, *N,N*-dimethylformamide (DMF), 4-dimethylaminopyridine
4 (DMAP), sodium chloride, alamarBlue[®], Texas Red[®] hydrazide, LysoTracker[®] Red DND-99
5 and Hoechst 33342 were obtained from Fisher Scientific (Loughborough, UK). L-lysine
6 methyl ester dihydrochloride, *N,N'*-dicyclohexylcarbodiimide (DCC) and ninhydrin were
7 purchased from Alfa Aesar (Heysham, UK). Anhydrous ethanol, acetone, hydrochloric acid,
8 potassium carbonate, sodium hydroxide, chloroform, methanol, diethyl ether, sodium citrate
9 dehydrate, triethylamine and sodium phosphate were obtained from VWR (Lutterworth, UK).
10 Defibrinated sheep red blood cells (RBCs) were purchased from TCS Biosciences Ltd
11 (Buckingham, UK).
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25 2.2. Synthesis of PLP and Comb-Like Polymers

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27 Poly(L-lysine *iso*-phthalamide) (PLP) was synthesized according to the previously
28 published method.²¹ NDA was conjugated onto the pendant carboxylic acid moieties along
29 the PLP backbone (designated as PLP-NDA) at various degrees of substitution via
30 DCC/DMAP mediated amide coupling chemistry (Scheme 1). Briefly, PLP (3 g) and DMAP
31 (0.6 g, 20 wt% of PLP) were dissolved in 60 mL anhydrous DMSO/DMF (1:3 v/v). A
32 specific amount of NDA was dissolved in 2 mL of chloroform and then transferred to the
33 reaction solution, to which was added dropwise DCC (3 molar equivalents of NDA) in 10 mL
34 anhydrous DMF. The reaction was monitored by thin-layer chromatography
35 (CHCl₃:MeOH:triethylamine = 8:2:0.2, using ninhydrin to visualize amine). Solid impurities
36 were removed by vacuum filtration and the reaction solution was added with 20 mL 5 wt%
37 NaOH in anhydrous ethanol and then precipitated rapidly into five volumes of diethyl ether.
38 The precipitate was collected and re-dissolved in deionized water. 0.2 M HCl solution was
39 then used to precipitate the polymer out. The precipitate was collected by vacuum filtration
40 and re-dissolved in deionized water with 0.2 M NaOH. The precipitation-filtration-
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redissolution process was repeated twice to remove inorganic salts and residual organic solvents. The polymer was further purified by dialysis against deionized water in a Visking dialysis tubing (Medicell, UK, MWCO 12-14 kDa) to remove oligomers. After dialysis, the polymer solution was titrated to pH 7.4 using 0.2 M NaOH and then lyophilized to a fine white powder. To prepare its acidic form, the dialyzed polymer solution was acidified to around pH 3.0 using 0.2 M HCl. The precipitate was collected by vacuum filtration and lyophilized.

Scheme 1. Synthesis of poly(L-lysine *iso*-phthalamide)s grafted with NDA (PLP-NDA).

2.3. Structural Characterization

The polymers in acidic form were dissolved in d^6 -DMSO and their ^1H -NMR spectra were obtained on a 400 MHz NMR spectrometer (Bruker, Germany) at room temperature (Figure S1). The ratio of the integral 0.77-0.91 ppm to the integral 7.45-7.64 ppm was used to calculate the degree of grafting $n\%$, which denotes to the actual molar percentage of NDA relative to the pendant carboxylic acid groups along the backbone of PLP. The molecular weight and polydispersity index (PDI) of PLP ($M_w = 35.7$ kDa, $M_n = 17.9$ kDa, PDI = 1.99) were determined according to the previous publication.¹⁹ The degrees of grafting obtained from ^1H -NMR were used to calculate the molecular weights of the comb-like pseudopeptidic polymers, *e.g.* PLP-NDA 3% ($M_w = 36.3$ kDa, PDI = 1.99), PLP-NDA 10% ($M_w = 37.7$ kDa,

PDI = 1.99) and PLP-NDA 18% ($M_w = 39.3$ kDa, PDI = 1.99). The FT-IR spectra of PLP-NDA in acid form were recorded on a Spectrum 100 FT-IR Spectrometer (Perkin Elmer, USA) (Figure S2), with characteristic peaks located at 3292 cm^{-1} (N–H str and O–H str), 1724 cm^{-1} (C=O acid str), 1629 cm^{-1} (amide band I), and 1529 cm^{-1} (amide band II).

2.4. Turbidimetry

Polymer solutions were prepared in phosphate or citrate buffer (100 mM) at specific pHs and allowed to equilibrate for 48 h. The transmittance of the polymer solutions at different pHs was measured using a Genesys 10S UV-vis spectrophotometer (Thermo Scientific, UK) at 480 nm.

2.5. Fluorescence Spectroscopy

The hydrophobic pyrene has been previously employed as a probe to investigate the pH-induced hydrophobic association of polymers in aqueous solution.^{19,23} 1.0 mM pyrene stock solution was freshly prepared in absolute methanol and then added to each polymer solution at a specific pH to give a final pyrene concentration of 600 nM. The polymer solutions were equilibrated for 48 h with protection from light. The excitation spectra ($\lambda_{em} = 390\text{ nm}$) of pyrene dissolved in the polymer solutions were recorded on a FluoroMax spectrofluorometer (HORIBA, Japan). The ratio of the fluorescence intensities at 338 nm to 333 nm in the excitation spectra of pyrene, I_{338}/I_{333} , was then calculated. The critical aggregation concentrations (CACs) of the polymers were obtained from the concentration-dependent variations of I_{338}/I_{333} at pH 7.4.

2.6. Dynamic Light Scattering (DLS)

The hydrodynamic size and size distribution of the polymers in aqueous solution were investigated using a dynamic light scattering platform (Zetasizer Nano S, Malvern, UK). The polymer aqueous solutions were prepared in the buffer at specific pHs and equilibrated for 48 h. All the samples were filtered through 0.45 μm filters before measurements. The dynamic light scattering was then measured at a scattering angle of 137° in a 10-mm diameter cell at 25 $^\circ\text{C}$, repeated for eleven times for each run.

2.7. Hemolysis Assay

The membrane disruptive activity of the polymers was examined using a previously established hemolysis model.²⁴ Briefly, the polymer solutions at different concentrations were prepared in the 100 mM phosphate buffer or citric buffer at specific pHs. Defibrinated sheep RBCs were washed at least three times with 150 mM NaCl solution and resuspended in the polymer buffer solution to a final concentration of $1\text{--}2 \times 10^8$ RBCs mL^{-1} . Negative and positive controls were prepared by resuspending RBCs in buffer alone and deionized water respectively. The samples were incubated in a shaking water bath (120 rpm) at 37 $^\circ\text{C}$ for 1 h, and then centrifuged at 4000 rpm for 4 min. The absorbance of the supernatant at 540 nm was measured using the UV-vis spectrophotometer and relative hemolysis was calculated.

2.8. Laser Scanning Confocal Microscopy of non-nucleated RBCs

Laser scanning confocal microscopy of RBCs was performed to elucidate the mechanism of the polymer-induced cell membrane destabilization. RBCs were washed with 150 mM NaCl solution for three times, and then resuspended in the buffer solutions at specific pHs containing 10 μM FITC-dextran and 1 μM Texas Red[®] hydrazide (with or without the polymers) to a final cell concentration of 10^7 RBCs mL^{-1} . The cells were imaged using an

LSM-510 inverted laser scanning confocal microscope (Zeiss, Germany) at 37 °C. FITC and Texas Red[®] hydrazide were excited at 488 and 543 nm respectively.

2.9. Cell Culture

Two adherent epithelial cancer cell lines, HeLa derived from human cervical carcinoma and A549 derived from human lung carcinoma, were grown in DMEM supplemented with 10% (v/v) FBS and 100 U mL⁻¹ penicillin unless specified otherwise. The non-cancerous CHO adherent cell line derived from the ovary of the Chinese hamster were grown in DMEM supplemented with 1% MEM non-essential amino acids, 10% (v/v) FBS and 100 U mL⁻¹ penicillin unless specified otherwise. The cells were trypsinized using trypsin-EDTA and maintained in a humidified incubator with 5% CO₂ at 37 °C.

2.10. Alamar Blue Assay

The cytotoxicity of the polymers was evaluated by Alamar Blue assay. HeLa, CHO or A549 cells were seeded into a 96-well plate (Corning, USA) containing the complete DMEM (0.1 mL per well) at a density of 1×10^4 cells per well for 24 h. The spent medium was replaced with 0.1 mL of the 0.22 μm filter-sterilized serum-free DMEM containing the polymer at specific concentrations. After certain periods of incubation, the polymer-containing medium was replaced with the complete DMEM containing 10% (v/v) alamarBlue[®]. The plate was further incubated for 4 h according to the manufacturer's instructions and the fluorescence of each well was measured using a spectrofluorometer (GloMax[®]-Multi Detection System, Promega, USA) at the excitation wavelength of 525 nm and the emission wavelength of 580-640 nm. The cytotoxic effect was determined from the fluorescence readings.

2.11. Laser Scanning Confocal Microscopy of Nucleated Mammalian Cells

Calcein, known as a membrane-impermeable fluorophore, was employed to evaluate the ability of the comb-like polymers to release endocytosed molecules into the cytoplasm of different cells. 2 mL of HeLa, CHO or A549 cells (2×10^5 cells mL^{-1}) were seeded into a 35-mm glass-bottom culture dish (MatTek, USA) and cultured for 24 h. The spent medium was then replaced by 2 mL of the 0.22 μm filter-sterilized serum-free medium containing the polymer (0.5 mg mL^{-1}) and calcein (2 mg mL^{-1}). The cells were incubated with 2 mg mL^{-1} calcein alone as a control. After treatment with or without polymer for a specific period, the cells were washed three times with D-PBS and replenished with the complete DMEM for further incubation. LysoTracker (50 nM) and Hoechst 33342 (1 $\mu\text{g mL}^{-1}$) were employed to stain endosomal/lysosome and nuclei respectively for 5 min and then the cells were washed with D-PBS three times. The cells were imaged using the LSM-510 inverted laser scanning confocal microscope. Calcein was excited using a 488 nm laser and the emission at 535 nm was collected.

2.12. Statistical Analysis

All data points were repeated in triplicates ($n = 3$). Reported results and graphical data are mean values with standard deviation encompassing a 95% confidence interval.

3. RESULTS AND DISCUSSION

3.1. Aqueous Solution Properties

The pH-mediated phase separation of the comb-like PLP-NDA polymers bearing different amounts of the hydrophobic relatively long side chains was determined at polymer concentration of 1.0 mg mL^{-1} . The pH value at the onset of precipitation (pH_p), at which the transmittance started to decrease, can be manipulated by varying the degree of substitution

with NDA. As shown in Figure S3, compared with the linear counterpart PLP, the comb-like polymers with a degree of substitution ≤ 10 mol%, *e.g.* PLP-NDA 3% and PLP-NDA 10%, displayed a negligible change in pH_p . With further increasing the molar percentage of NDA moieties, the pH_p value increased from 4.5 for PLP to 5.0 for PLP-NDA 18%. This suggests that incorporation with hydrophobic NDA side chains can result in enhanced polymer hydrophobicity. This is consistent with the report that the increased amount of hydrophobic amino acid pendant groups increased the hydrophobicity of polymer.^{19,23}

The poorly water-soluble pyrene was used as a probe to investigate the pH-mediated hydrophobic association of the polymers. The ratio of fluorescence intensities at 338 to 333 nm in the excitation spectra of pyrene (I_{338}/I_{333}) increases upon its transfer into a less polar environment.²³ Herein, the changes in I_{338}/I_{333} were used to evaluate the effects of the relatively long aliphatic side chains and the polymer concentration on the hydrophobic association of the comb-like polymers. Figure 1A shows the changes of I_{338}/I_{333} as a function of pH in the aqueous solutions of PLP, PLP-NDA 3%, PLP-NDA 10% and PLP-NDA 18% at the concentration of 0.5 mg mL⁻¹. The pH at the onset of hydrophobic association (pH_h), defined as the pH value where I_{338}/I_{333} started to increase, was 4.8 for the linear PLP polymer. At $\text{pH} \geq 4.8$, its I_{338}/I_{333} value was almost constant at around 0.6, indicating that pyrene was in a hydrophilic environment. When the pH decreased below 4.8, a pronounced increase in I_{338}/I_{333} was observed, indicative of the transfer of pyrene to an increasingly hydrophobic environment. The rapid pH-mediated changes in I_{338}/I_{333} can be ascribed to a pH-dependent coil-to-globule transition of polymer conformation.²⁵ At pH 7.4, the polymer displayed an expanded structure due to the electrostatic repulsion between negatively charged carboxylate ions. Upon acidification, the carboxylate groups were protonated and hydrophobic association became predominant, leading to the formation of a compact structure with the hydrophobic core where pyrene was preferentially solubilized.¹⁹

The pH-mediated hydrophobic association was enhanced by incorporating the relatively long aliphatic side chains onto the PLP backbone. As shown in Figure 1A and Table 1, with increasing the degree of substitution up to 10 mol%, the comb-like polymers (*e.g.* PLP-NDA 3% and PLP-NDA 10%) showed a gradual increase in I_{338}/I_{333} , with a concomitant increase in pH_h and broadening in the pH range for hydrophobic association. A further increase in the degree of substitution to 18 mol% (*e.g.* PLP-NDA 18%) led to the almost constant I_{338}/I_{333} value at ~ 1.5 , suggesting the localization of pyrene in the hydrophobic core of compact aggregates that were formed throughout the pH range tested. The enhanced hydrophobic association of the comb-like PLP-NDA polymers compared to their linear counterpart PLP can be ascribed to the enhanced hydrophobicity resulting from grafting with hydrophobic NDA pendant chains. It is consistent with the observation that grafted aliphatic chains can lead to enhanced aggregation of poly(aspartic acid).²⁶

Figure 1. (A) pH-dependent variations in I_{338}/I_{333} of PLP (■), PLP-NDA 3% (●), PLP-NDA 10% (▲) and PLP-NDA 18% (▼) at 0.5 mg mL⁻¹ in 100 mM phosphate or citrate buffer. (B) Concentration-dependent variations in I_{338}/I_{333} of PLP (■), PLP-NDA 3% (●), PLP-NDA 10% (▲) and PLP-NDA 18% (▼) at pH 7.4 in 100 mM phosphate buffer.

Table 1. The pH values at the onset of hydrophobic association (pH_h) and pH ranges for hydrophobic association of PLP and the comb-like PLP-NDA polymers at 0.5 mg mL⁻¹ and their critical aggregation concentrations (CACs) at pH 7.4.

	PLP	PLP-NDA 3%	PLP-NDA 10%	PLP-NDA 18%
pH _h	4.8 ± 0.2	5.0 ± 0.2	6.0 ± 0.2	N/A
pH range	3.5 – 4.8	4.0 – 5.0	3.5 – 6.0	N/A
CAC (mg mL ⁻¹)	N/A	0.342	0.282	0.031

The amphiphilic nature of the comb-like PLP-NDA polymers provides an opportunity for self-assembling into multimolecular aggregates. The variations in I_{338}/I_{333} as a function of polymer concentration at pH 7.4 were employed to determine the CAC (Figure 1B). The CAC value was obtained from the intersection of the line through the points on the rapidly varying part and the line through the points at the low-concentration extreme of the plot. Above the CAC, the polymers tended to form multimolecular aggregates due to the predominant intermolecular interactions. As summarized in Table 1, the CAC value of the comb-like polymer decreased with increasing the molar percentage of NDA grafts, which could be attributed to the enhanced polymer hydrophobicity.

The hydrodynamic size of the pseudopeptidic polymers at 0.5 mg mL⁻¹ was measured by DLS. Both the comb-like PLP-NDA polymers and their linear counterpart PLP displayed two size populations at pH 7.4. As shown in Figure 2, PLP had a small particle population with a

mean size of 10.3 ± 2.3 nm and a large population with a mean size of 384.2 ± 34.5 nm. With increasing the grafting density of NDA side chains up to 18 mol%, the mean size of the small population remained similar, whilst the mean size of the large population was dramatically reduced to 51.9 ± 1.6 nm. This was due to the enhanced intermolecular and intramolecular interactions resulting from incorporation of hydrophobic side chains.^{26,27}

The size change of the pseudopeptides as a function of pH was also depicted in Figure 2. At pH 7.4 both PLP and PLP-NDA 18% formed loose aggregates, due to the predominant electrostatic repulsion between negatively charged carboxylate groups²⁸, and thus displayed two size populations. Upon acidification to pH 5.5, PLP retained two size populations, but the mean particle size of its large population was reduced considerably from 384.2 ± 34.5 to 86.0 ± 10.5 nm (Figure 2A). This suggests that protonation of the carboxylate groups caused the enhanced hydrophobic interactions and the consequent formation of compact aggregates. As comparison, PLP-NDA 18% displayed only one small particle population with a mean hydrodynamic size of 16.4 ± 5.6 nm at pH 5.5 (Figure 2B). This was because the presence of relatively long aliphatic side chains in the comb-like polymer considerably enhanced hydrophobic interactions, which became predominant and led to the formation of a more compact structure with a single size population. It has been previously reported that the amphiphilic pseudopeptidic polymer PLP bearing poly(ethylene glycol) displayed a similar transition from a bimodal size distribution to a single-modal distribution upon acidification.²⁸

Figure 2. Particle size distribution of (A) PLP and (B) PLP-NDA 18% at 0.5 mg mL⁻¹ at pH 7.4 (solid) and pH 5.5 (dash).

3.2. Cell Membrane Disruption

The cell membrane disruptive activity of the comb-like polymers was assessed using RBC membranes as a model for endosomal membranes.²⁴ The pH range of 7.4 – 4.5 for the hemolysis assay mimics the gradually acidifying pH environment in early endosomes, late endosomes and lysosomes during the endocytic pathway.²⁹ Figure 3A shows the pH-dependent hemolysis of PLP and the comb-like PLP-NDA polymers at 0.5 mg mL⁻¹. PLP only displayed limited membrane disruption at pH below 5.5. In contrast, the comb-like polymers containing the hydrophobic relatively long NDA side chains exhibited negligible or limited hemolysis at physiological pH, but exhibited significantly enhanced hemolytic activity upon acidification compared to their linear counterpart. The maximum degree of hemolysis was increased from 28.8 ± 0.9% for PLP to 42.3 ± 0.1%, 77.1 ± 3.7% and 99.6 ± 0.2% for PLP-NDA 3%, PLP-NDA 10% and PLP-NDA 18% respectively. The pH for the maximum hemolysis was also increased from a lysosomal pH 4.5 for PLP to an endosomal pH 5.5 for the three PLP-NDA polymers. It is also worth noting that increasing the degree of grafting with NDA resulted in the onset of hemolytic activity at a higher pH value, which as a

consequence enabled the comb-like polymers to exhibit significantly enhanced hemolytic activity within the pH range (6.0 – 6.8) typical of early endosomes²⁴ (reaching a hemolysis of $78.3 \pm 0.2\%$ for PLP-NDA 18%). With acidification to $\text{pH} < 5.5$, the hemolysis efficiency decreased due to precipitation of the comb-like polymers. A similar effect has been reported for the hemolytic activity of the pseudopeptidic polymers bearing pendant hydrophobic natural amino acids.²⁴

The improved cell membrane destabilizing activity of the PLP-NDA polymers could be due to their comb-like structure and enhanced hydrophobicity. The ranking of hemolytic activity at endosomal pH amongst the polymers was $\text{PLP} < \text{PLP-NDA } 3\% < \text{PLP-NDA } 10\% < \text{PLP-NDA } 18\%$. This ranking well correlated with the NDA graft number and polymer hydrophobicity. Vial *et al.* have reported that amphiphilic polymers lacking hydrophobic side-chain moieties can not effectively bind or disrupt the vesicular membrane.³⁰ It has also been shown that the hydrophobic association of poly(α -ethylacrylic acid) containing short ethyl pendant groups can rapidly overcome the charge repulsion and therefore exhibited the improved membrane binding.³¹ Other researchers have reported that even a small number of long alkyl side chains can facilitate the deeper insertion of poly(*N*-isopropylacrylamides) derivatives into the liposomal bilayer.³²

The ability of PLP-NDA 18% to hemolyze RBCs at endosomal pH 5.5 and physiological pH was further evaluated as a function of polymer concentration (Figure 3B). PLP-NDA 18% showed limited hemolysis at pH 7.4 within the concentration range tested ($0.005 - 10 \text{ mg mL}^{-1}$), but exhibited the dilution-independent, excellent membrane disruptive activity at pH 5.5 over a wide concentration range ($0.5 - 10 \text{ mg mL}^{-1}$). When diluting the polymer concentration 400-fold to as low as 0.025 mg mL^{-1} , PLP-NDA 18% still retained a high level of hemolysis of $72.9 \pm 0.5\%$ at pH 5.5. The dilution-independent characteristic of the comb-like polymer may be attributed to its stable membrane anchoring, which resulted from the

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3 deep insertion of long hydrophobic side chains into the membrane.³³ This dilution resistance
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5 is of critical importance for drug delivery applications since after administration, the
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7 concentration of drug delivery systems could be considerably decreased due to the dilution
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9 effect during circulation in the bloodstream.³⁴ This profile of membrane activity at both
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11 physiological and endosomal pH is desirable for intracellular drug delivery applications.
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15 The kinetics of hemolysis induced by the comb-like polymers at 0.5 mg mL⁻¹ were also
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17 investigated at pH 5.5. As shown in Figure 3C, after only 10 min of incubation, 72.8 ± 1.8%
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19 of RBCs were hemolyzed by PLP-NDA 18%, followed by almost complete membrane
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21 destabilization after 20 min. As comparison, the polymers with ≤ 3% NDA showed not only
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23 lower membrane disruption but also slower kinetics. The presence of PLP-NDA 3% resulted
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25 in the maximum degree of hemolysis of 47.1 ± 4.0% after 50 min, and no hemolysis was
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27 detected for the linear PLP within the time period tested at pH 5.5. This indicates that
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29 increasing the hydrophobicity and tuning of the comb-like architecture of the polymers could
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31 result in a stronger driving force to rapidly and effectively overcome the electrostatic
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33 repulsion from the negatively charged cell membrane,³⁴ leading to faster membrane binding
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35 and greater membrane disruption. The fast and efficient membrane destabilization of the
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37 comb-like polymers, in particular PLP-NDA 18%, would enable them to disrupt the
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39 endosomal membrane before the vesicular evolution from endosomes to lysosomes during
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41 the endocytic pathway, which has been reported to take several hours.³⁵
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Figure 3. Relative hemolysis of RBCs in the presence of PLP and the comb-like PLP-NDA polymers. (A) pH-dependent hemolysis of RBCs incubated with PLP (■), PLP-NDA 3% (●), PLP-NDA 10% (▲) and PLP-NDA 18% (▼) at 0.5 mg mL^{-1} for 1 h. (B) Concentration-dependent hemolysis of RBCs incubated with PLP-NDA 18% at pH 7.4 (open column) and pH 5.5 (closed column). (C) Time-dependent hemolysis of RBCs incubated with PLP (■), PLP-NDA 3% (●), PLP-NDA 10% (▲), PLP-NDA 18% (▼) at 0.5 mg mL^{-1} at pH 5.5.

3.3. Mechanism of Cell Membrane Disruption

Lipid membrane disruption could be caused by membrane fusion³⁶, pore formation³⁷ or membrane solubilization¹². In order to investigate the mechanism of hemolysis by the comb-like polymers, the RBC membrane integrity was examined by laser scanning confocal microscopy. As shown in Figures S4 and Figure 4, FITC-dextran ($M_w = 4$ kDa) and Texas Red[®] hydrazide could not pass through the RBC membrane treated with buffer only (at both pH 7.4 and 5.5), 0.5 mg mL⁻¹ PLP (at both pH 7.4 and 5.5), or 0.5 mg mL⁻¹ PLP-NDA 18% (at pH 7.4). However, when the RBCs were incubated with 0.5 mg mL⁻¹ PLP-NDA 18% at pH 5.5, more than 90% of the cells were turned into erythrocyte ghosts (Figure 4). The membrane of erythrocyte ghosts was brightly stained with Texas Red[®] hydrazide, which could react with peroxidized lipids during the formation of ghost cells to give bright red fluorescence.³⁸ The FITC-dextran 4k was observed inside the erythrocyte ghosts and there was no difference in fluorescence intensity between the inside and the outside, indicating that the FITC-dextran 4k efficiently permeated through the membrane. No membrane fusion or solubilization was observed and the membrane structure was retained as shown by Texas Red[®] hydrazide staining, further confirming that the comb-like polymer induced pore formation and hemolysis without membrane rupture at endosomal pH. This is different from the hemolysis mechanism of poly(α -ethylacrylic acid) bearing pendant hydrophobic short ethyl groups, which could burst the cell membrane as a result of pore formation.³⁹

Membrane binding and insertion are regarded as two important continuous steps for the polymer-mediated lipid membrane destabilization.³³ The electrostatic repulsion between the anionic polymers and the negatively charged cell membrane could prevent the effective membrane binding and insertion of the polymers. Although the linear parent polymer PLP displayed a coil-to-globule conformational change at acidic pH²², no or low hemolysis was observed for PLP at 0.5 mg mL⁻¹ within the pH range tested (from pH 7.4 to 4.5) due to the

limited polymer-membrane interaction. For the comb-like PLP-NDA polymers bearing hydrophobic relatively long side chains, the largely increased hydrophobic interaction could outweigh the electrostatic repulsion. Furthermore, the hydrophobic side chain could act as a membrane anchor⁴⁰, leading to the pore formation in the lipid membrane upon pH-mediated conformational change at acidic pHs.

Figure 4. Confocal microscopy fluorescence and differential interference contrast (DIC) images of RBCs incubated with 10 μM FITC-dextran ($M_w = 4K$ Da) and 1 μM Texas Red® hydrazide in the absence or presence of the comb-like polymer PLP-NDA 18% and its linear counterpart PLP (0.5 mg mL^{-1}) at pH 5.5 for 30 min. Scale bar 10 μm.

Figure 5 depicts the RBCs incubated with different sized FITC-dextran ($M_w = 4k - 2000k$ Da) in the presence of 0.5 mg mL^{-1} PLP-NDA 18% at pH 5.5 for 30 min. FITC-dextran with

the molecular weight up to 70k Da efficiently entered the resulted erythrocyte ghosts. The pore size formed by PLP-NDA 18% was therefore estimated to be approximately 6 nm from the Stoke's radius of FITC-dextran 70k according to the supplier's information. The successful transport of FITC-dextran into RBCs at endosomal pH implies that the novel comb-like polymer can destabilize the endosomal membrane to efficiently release endocytosed macromolecules into the cytoplasm without totally lysing vesicular compartments. It is ideal for cytoplasmic drug delivery through endosomal-lysosomal trafficking pathways because by achieving this cytotoxicity can be minimized.

Figure 5. Confocal microscopy fluorescence and DIC images of RBCs incubated with 1 μM Texas Red[®] hydrazide and 10 μM FITC-dextran with different molecular weights in the presence of 0.5 mg mL^{-1} PLP-NDA 18% at pH 5.5 for 30 min. Scale bar 10 μm .

3.4. *In-vitro* Cytotoxicity

The concentration- and time-dependent cytotoxic effects of the comb-like polymer PLP-NDA 18% toward HeLa (Figure 6A), CHO (Figure 6B) and A549 cells (Figure 6C) were assessed by Alamar Blue assay. PLP-NDA 18% displayed no or limited cytotoxicity toward all the three cell lines within the concentration range of 0.005 – 0.5 mg mL^{-1} after treatment for up to 48 h. With further increasing the polymer concentration from 1.0 to 10.0 mg mL^{-1} , HeLa, CHO and A549 showed different levels of tolerance to PLP-NDA 18%. The IC_{50} (*i.e.* the concentration leading to inhibition of 50% cell viability) of PLP-NDA 18% against HeLa after 12 h of treatment was $2.94 \pm 0.28 \text{ mg mL}^{-1}$, while it decreased to 1.59 ± 0.22 and $1.05 \pm 0.14 \text{ mg mL}^{-1}$ after extended treatment for 24 h and 48 h respectively. CHO and A549 cells demonstrated the increasingly better tolerance than HeLa cells, with an increase in IC_{50} to 2.81 ± 0.95 and $4.51 \pm 0.06 \text{ mg mL}^{-1}$ respectively after 48 h of treatment. Compared with the cytotoxic effects of its linear counterpart PLP (Figure S5), the comb-like polymer PLP-NDA 18% did not exhibit significant changes in the viability of HeLa, CHO and A549 cells within the polymer concentration range (0.005 – 1.0 mg mL^{-1}) after treatment for up to 48 h. This indicates that incorporation of the hydrophobic relatively long NDA side chains onto the PLP backbone did not induce considerable cytotoxicity. When the polymer concentration was sufficiently high (5.0 – 10.0 mg mL^{-1}), PLP-NDA 18% displayed a relatively higher cytotoxicity than PLP.

Figure 6. Relative viabilities of (A) HeLa, (B) CHO and (C) A549 cells incubated with PLP-NDA 18% at various concentrations for 4 h (blank columns), 12 h (grey columns), 24 h (black columns) and 48 h (striped columns).

3.5. Release of Endocytosed Materials from Intracellular Vesicles

The potential of the comb-like PLP-NDA polymers to release endocytosed materials into the cytoplasm of different cell lines, such as human cervical carcinoma HeLa cells (Figure

7A), human lung carcinoma A549 cells (Figure 7B) and non-cancerous CHO cells (Figure 7C), were assessed using membrane-impermeable calcein as a model drug. In the absence of polymer (the control), bright punctate spots were observed in all the three cell lines (Figure S6). This was due to the entrapment of the membrane impermeable fluorophore within intracellular vesicles after endocytosis²⁴. As shown in Figure S6, cotreatment with 0.5 mg mL⁻¹ PLP and 2.0 mg mL⁻¹ calcein resulted in a similar calcein-release profile compared to the control. As comparison, in Figure 7, the presence of 0.5 mg mL⁻¹ PLP-NDA 18% led to diffuse green fluorescence spreading over all the three cell lines, indicating the comb-like polymer mediated release of endocytosed calcein into the cytoplasm and the applicability of the system in various cancerous and non-cancerous cells. The ability to significantly enhance the release of endocytosed materials into the cytoplasm through pH-mediated membrane disruption, together with its no or low cytotoxicity, suggests the promising applications of the comb-like PLP-NDA polymer in cytoplasmic delivery of therapeutic agents.

Figure 7. Confocal microscopy images of (A) HeLa, (B) A549 and (C) CHO cells showing the subcellular distribution of calcein fluorescence. The cells were cotreated with 2.0 mg mL^{-1} calcein and 0.5 mg mL^{-1} PLP-NDA 18%. Images were acquired after 1 h of uptake and 3 h of further incubation in fresh DMEM for HeLa and CHO cells (2 h of uptake and 2 h of further incubation for A549 cells). Scale bar: $10 \text{ }\mu\text{m}$.

4. CONCLUSIONS

pH-responsive, comb-like PLP-NDA polymers have been synthesized by grafting hydrophobic relatively long NDA alkyl chains onto the backbone of a linear pseudopeptide, PLP, at different degrees of substitution. The pH-responsive changes in conformation, hydrodynamic size and hemolytic activity could be manipulated by controlling the amount of NDA side-chain moieties. The significantly enhanced hydrophobicity and comb-like structure enabled the optimal polymer PLP-NDA 18% to have a vastly superior membrane-disruptive ability at endosomal pH compared to its linear counterpart PLP. At 0.5 mg mL^{-1} , PLP-NDA

18% caused complete hemolysis of RBCs at pH 5.5, while 0.5% hemolysis was achieved for its linear counterpart PLP under the same condition. The excellent membrane disruption of the comb-like polymer at endosomal pH was dilution-independent. The formation of ghost cells indicates the pore formation mechanism of cell membrane disruption. The comb-like polymer showed no or limited cytotoxicity at physiological pH. Its ability to efficiently release endocytosed materials into the cytoplasm of various cancerous and non-cancerous cell lines suggests its potential for cytoplasmic drug delivery applications.

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 ¹H-NMR and FT-IR spectra of PLP-NDA 18%, pH-dependent transmittance of PLP and various PLP-NDA polymers, confocal microscopy fluorescence images of RBCs incubated FITC-dextran and Texas Red® hydrazide in the absence or presence of PLP and PLP-NDA 18% at pH 7.4, relative viabilities of HeLa, CHO and A549 cells treated with PLP, confocal microscopy images of HeLa, A549 and CHO cells treated with calcein only or both calcein and PLP.

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Author Contributions

R.C. designed and supervised the research; S.C. performed all of the research with help from S.W., M.K. and M.B.; S.C. and R.C. analyzed data; S.C. and R.C. wrote the paper. All authors have read, commented on, and approved the final version of the paper.

Notes

The authors declare no competing financial interest.

DATA ACCESS STATEMENT

Data underlying this article can be accessed on Zenodo (www.zenodo.org) at DOI: 10.5281/zenodo.237043, and is openly available under the Creative Commons CC-BY license.

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