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Synthetic pH-Responsive Polymers for Protein Transduction

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Amphiphilic polymers containing weakly ionizable carboxyl groups and hydrophobic alkyl side chains can mimic the cell-transduction function of viral peptides.^[1] Such polymers display a change of conformation from extended chains to collapsed, hydrophobically stabilized globular structures in response to acidification below their pK_a ranges.^[2] This change leads to increased binding of polymers to eukaryotic cell membranes and subsequent membrane disruption.^[3-5] The pH-mediated conformational change depends principally on the balance between the hydrophobic interaction associated with hydrophobic moieties and the electrostatic repulsion resulting from charged carboxyl groups.^[6] The hydrophobicity of pendant hydrophobic groups is one of the factors that influence the conformational behavior of pH-responsive polymers.^[3,6,7] 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 increase endosomal membrane lysis activity.^[8-12]

Eccleston et al.^[13-15] described a biodegradable, metabolite-derived polyamide, poly(L-lysine *iso*-phthalamide), with a hydrophobic backbone and pendant carboxyl groups. Despite lacking hydrophobic side chains presented in fusogenic peptides and poly(α -alkylacrylic acid)s, poly(L-lysine *iso*-phthalamide) displays a pH-dependent conformational change^[16-18] and weak cell-membrane lytic capacity over a low pH range (pH 4.6–5.0).^[19,20] Recently, pH-responsive polymers have been synthesized by grafting hydrophobic amino acids—L-valine, L-leucine, and L-phenylalanine—onto the carboxylic acid moieties along the backbone of poly(L-lysine *iso*-phthalamide). The effects of hydrophobicity and structure of grafted side chains and degree of grafting on the pH responsiveness of polymer conformation and aggregation, cell-membrane-disruptive activity, in vitro cytotoxicity, and intracellular trafficking of the grafted polymers have been demonstrated and presented

elsewhere.^[21-23] Of these derivatives, the polymer with the optimal properties for endosomal membrane disruption is the phenylalanine derivatized polymer PP-75 at a stoichiometric degree of substitution of 75 mol% ($M_n = 24.9$ kDa, degree of grafting = 63.2%).^[21,22] In sheep erythrocyte models of endosomes, PP-75 was 35-fold more membrane disruptive on a molar basis than the highly lytic bee-sting peptide melittin at pH 6.5, within the pH range characteristic of early endosomes. PP-75 was not cytotoxic toward mammalian cells up to a polymer concentration of 2 mg mL^{-1} after 2 h of treatment. After 48 h, PP-75 had almost no cytotoxic effects at the concentration $< 0.125 \text{ mg mL}^{-1}$, while progressively decreased cell viability to 28% with increasing concentration up to 2 mg mL^{-1} .^[22]

Based on previous successful intracellular delivery of the model-drug calcein,^[22] it was thought that PP-75 may be able to deliver biological molecules (proteins and nucleic acids) through endosomal release. This study examines the ability of the synthetic polymer PP-75 to deliver Apoptin protein into mammalian cells. Apoptin, derived from chicken anemia virus (CAV), has a relative molecular mass of 13.6 kDa. Apoptin has been shown to induce apoptosis in more than 70 different tumor-cell lines, while leaving normal cells (primary fibroblasts, keratinocytes, smooth muscle cells, T cells, and endothelial cells) unharmed. Though the exact mechanism(s) are still under investigation, two key processes (tumor-specific phosphorylation and tumor-specific nuclear localization) are thought to play major roles in its unique activity.^[24] Furthermore, Apoptin-mediated apoptosis is induced in a p53 and Bcl-2 independent manner,^[25] rendering it a highly attractive therapeutic strategy for aggressive cancers. Exploitation of that strategy is, however, currently hampered by the lack of a clinically acceptable means of transporting Apoptin into cells. Currently, all studies of Apoptin delivery have been based on gene delivery or microinjection.^[24] This paper demonstrates the ability of PP-75 to mediate the transport of Apoptin into human osteogenic sarcoma Saos-2 cells. Here, Apoptin was used in the form of a fusion protein with maltose binding protein (MBP). MBP is routinely used to solubilize recombinant proteins with a proclivity for aggregation. In this case, the resulting MBP-Apoptin (MA, 55.8 kDa) is easily purified from bacterial cell extracts, whereas Apoptin alone forms insoluble inclusion bodies following translation in *Escherichia coli*. Noteborn and colleagues demonstrated that this recombinant protein exhibits biological activity nearly identical to ectopically expressed Apoptin.^[26] In all studies, MBP-Apoptin was used at a final concentration of $33 \mu\text{g mL}^{-1}$ ($0.6 \mu\text{M}$).

First, the uptake of FITC-labeled PP-75 (PP-75-FITC) into Saos-2 cells was studied. The Saos-2 cell line (ATCC HTB85) is derived from an osteogenic sarcoma from an 11-year-old Caucasian female that underwent chemotherapy.^[27] The cell line is p53 negative and serves as a model for tumors with missing or mutated p53. Flow-cytometry experiments were conducted to quantify the polymer uptake after 1 h incubation with PP-75-FITC. As expected, both the proportion of cells with internalized PP-75-FITC and amount of internalized PP-75-FITC increased with polymer concentration (data not shown). The proportion of viable Saos-2 cells with internalized PP-75-FITC increased from approximately 50% FITC +, at $12.5 \mu\text{g mL}^{-1}$ PP-75-FITC, to nearly 80% FITC +, at $100 \mu\text{g mL}^{-1}$ PP-75-FITC. A similar trend was observed for mean fluorescence, which increased in a nearly linear fashion from 12.5 to $50 \mu\text{g mL}^{-1}$ polymer. From 50 to $100 \mu\text{g mL}^{-1}$ polymer there was little increase in the mean fluorescence. These flow cytometry data were corroborated by visual evidence from confocal microscopy. Control cells incubated in culture medium only exhibited no fluorescence. Cells treated with $25 \mu\text{g mL}^{-1}$ PP-75-FITC for 1 h displayed a relatively low level of fluorescence, whereas cells treated with 50 and $100 \mu\text{g mL}^{-1}$ PP-75-FITC displayed a substantial increase in fluorescence.

The concentration dependence of MBP–Apoptin PP-75 interaction has been investigated (Fig. 1). Native polyacrylamide-gel electrophoresis (PAGE) assays were performed to examine if the addition of PP-75 retarded the movement of MBP–Apoptin under native electrophoresis conditions. As seen from the polymer-only lanes in Figure 1A, high concentrations ($700\text{--}1000\text{ }\mu\text{g mL}^{-1}$) resulted in a large polymer structure that ran near $75\text{--}250\text{ kDa}$, indicating concentration-dependent hydrophobic association of the polymer. The broad staining in these lanes may be due to the formation of variable-sized hydrophobic aggregates.^[21]

In vitro, MBP–Apoptin spontaneously forms soluble, non-covalent, globular complexes approximately 80 nm in diameter.^[28] Attributed to the N-terminal portion of Apoptin and stabilized by hydrophobic interactions, these complexes are composed of $30\text{--}40$ MBP–Apoptin subunits,^[28] and have estimated molecular weight of approximately $2.5 \pm 0.3\text{ MDa}$.^[29] This multimerization of MBP–Apoptin accounts for its minimal migration in the leftmost lane of Figure 1A. Interestingly, the concentration of PP-75 in Figure 1A was sufficient to dissociate the multimeric MBP–Apoptin complex, and resulted in a sharp band near 48 kDa (long arrow). MBP–Apoptin runs at 55.8 kDa band under denaturing conditions,^[29] so 48 kDa is within the error ($\sim 15\%$) of using Native PAGE for molecular-weight estimation. Complex dissociation is likely due to intercalation and solubilization of multimeric MBP–Apoptin globules by PP-75, enabling the migration of individual MBP–Apoptin subunits through the gel. The three faint bands surrounding MBP–Apoptin can be ascribed to three expression products (short arrows) consistently copurified with MBP–Apoptin.^[29] Based on the broad staining pattern near the top of the gel, PP-75 was unable to completely dissociate the MBP–Apoptin complex.

In Figure 1B, PP-75 concentration was not sufficient to dissociate the multimeric MBP–Apoptin complexes. The presence of $100\text{ }\mu\text{g mL}^{-1}$ PP-75 resulted in wider staining pattern than MBP–Apoptin alone, but did not induce significant change in the protein migration. The effect is similar, though less pronounced, as the polymer concentration decreases. This indicates inefficient and incomplete dissociation of MBP–Apoptin complexes at lower PP-75 concentrations. The critical concentration at which PP-75 can dissociate MBP–Apoptin complexes has yet to be confirmed, but preliminary experiments indicate this is near $400\text{--}600\text{ }\mu\text{g mL}^{-1}$ PP-75 for $33\text{ }\mu\text{g mL}^{-1}$ MBP–Apoptin.

Flow-cytometry experiments were conducted to examine the ability of PP-75 to facilitate delivery of fluorescent MBP–Apoptin conjugated with Alexa Fluor 647 (MA-AF647) to Saos-2 cells (Fig. 2). As shown in Figure 2A, a majority of Saos-2 cells were able to internalize the fluorescent protein, irrespective of PP-75-FITC concentration. In cells treated with $0.6\text{ }\mu\text{M}$ naked MA-AF647, nearly 90% exhibited protein uptake. The amount increased slightly to approximately 98% upon the addition of PP-75-FITC, even at a dilute concentration of $5\text{ }\mu\text{g mL}^{-1}$. The mean fluorescence MA-AF647 in viable cells, shown in Figure 2B, demonstrates that low concentrations of PP-75-FITC are able to enhance the uptake of constitutively internalized MA-AF647, although low concentrations of the polymer ($100\text{ }\mu\text{g mL}^{-1}$) were found insufficient to dissociate the multimeric MBP–Apoptin complexes (Fig. 1B). The presence of $5\text{ }\mu\text{g mL}^{-1}$ PP-75-FITC resulted in mean fluorescence two-fold greater than naked MA-AF647. This amount increased further in the presence of $10\text{ }\mu\text{g mL}^{-1}$ PP-75-FITC.

The effect of increasing polymer concentration to moderate (25 and $100\text{ }\mu\text{g mL}^{-1}$) and high (500 and $1000\text{ }\mu\text{g mL}^{-1}$) levels on MA-AF647 delivery was also investigated. Again, MA-AF647 was internalized in nearly all-viable cells, as shown in Figure 2C. In samples treated with $0.6\text{ }\mu\text{M}$ naked MA-AF647, nearly 85% of cells demonstrated MA-AF647 uptake. The presence of PP-75-FITC increased this value to nearly 100% . Despite uptake by a large

proportion of Saos-2 cells (Fig. 2C), naked MA-AF647 does not accumulate in cells to an appreciable extent, as shown by the minimal increase in fluorescence relative to control in Figure 2D. However, incorporation with the polymer resulted in a significant increase in the amount of internalized MA-AF647 as the mean fluorescence of cells treated with $25 \mu\text{g mL}^{-1}$ PP-75-FITC and $33 \mu\text{g mL}^{-1}$ MA-AF647 increased nearly eight-fold relative to naked MA-AF647. Similarly, samples treated with $100 \mu\text{g mL}^{-1}$ PP-75 demonstrated a seven-fold increase in mean fluorescence of MA-AF647 relative to naked MA-AF647. Further increase in polymer concentration to 500 and $1000 \mu\text{g mL}^{-1}$ PP-75 resulted in a decreased ability to deliver MA-AF647, and the level of protein fluorescence in samples treated with $1000 \mu\text{g mL}^{-1}$ PP-75-FITC was comparable to that of naked MA-AF647. This may possibly arise from dissociation of multimeric MBP–Apoptin complexes observed only at high polymer concentrations (Fig. 1A), and suggests an optimum protein/polymer ratio for efficient protein transduction.

These flow-cytometry data were validated by studying the intracellular distribution and colocalization of MA-AF647 and PP-75-FITC using confocal microscopy. Figure 3 displays the evidence that PP-75 both enhances uptake and facilitates cytoplasmic release of MA-AF647. The image in Figure 3A shows that the cells are treated with $100 \mu\text{g mL}^{-1}$ PP-75-FITC alone. The diffuse staining pattern in the FITC channel is indicative of efficient release into the cytoplasm.^[22,30] The overlay image verifies that the diffuse staining is present within the cell membrane. Samples treated with MA-AF647 alone displayed evidence of the constitutive low-level uptake of naked protein observed in flow-cytometry experiments. The cells treated with MA-AF647 alone contained faint spots in the MA-AF647 channel (data not shown), displaying evidence of the constitutive low-level uptake of naked protein observed in flow-cytometry experiments. The punctate staining pattern suggests that naked MA-AF647 remains trapped in vesicular compartments.^[22,30] Viable cells incubated with unconjugated Alexa Fluor 647 did not show any fluorescence (data not shown). Nuclear localization is a key step that precedes apoptosis induced by Apoptin.^[31] As shown in Figure 3B and C, confocal microscopy indicates that MBP–Apoptin may translocate to the nucleus following successful PP-75-mediated endosomal release. Comparatively, Figure 3C, corresponding to 5 h further incubation, seems to have more punctate staining. Given MBP–Apoptin's proclivity to form large aggregates, comprised of 30–40 monomers, the red punctate staining may simply be MBP–Apoptin aggregation.^[29] Also, there are reports that MBP–Apoptin forms higher-order complexes with DNA,^[28] and this aggregation may increase with time. Although the extent of nuclear localization has not been determined, evidence of its possibility represents an exciting step toward the delivery of functional MBP–Apoptin.

The work presented here lays the basis for a novel protein-delivery system using the pH-responsive polymer PP-75. It describes the first-known successful cytoplasmic delivery of MBP–Apoptin using a synthetic-polymer drug-delivery system. The efficient cytoplasmic release represents a promising first step in the implementation of Apoptin as a viable antitumor therapeutic. Preliminary research has been conducted to confirm functional activity of MBP–Apoptin delivered by PP-75. After treatment of Saos-2 cells with $33 \mu\text{g mL}^{-1}$ MBP–Apoptin and $100 \mu\text{g mL}^{-1}$ PP-75 for 3 h followed by 24 h further incubation, flow-cytometry analysis suggested an approximately 30% increase in the population of cells found in the mid-apoptotic state, as compared to either MBP–Apoptin or PP-75 alone (data not shown). The mid-apoptotic state is characterized by lower DNA content (hypodiploid) and presence of fragmented DNA, which is a key feature of apoptosis. Further studies are underway to enhance functional activity of apoptosis-inducing MBP–Apoptin mediated by PP-75.

Experimental

Protein Preparation

Cloning, expression, and purification of the MBP–Apoptin fusion protein has been described previously [29]. Briefly, the Apoptin open reading frame (ORF) was cloned into a pMalTB plasmid, which codes for MBP. A coding sequence was inserted to link the C-terminal end of MBP and N-terminus of Apoptin with aspartate residues and a thrombin consensus site [29]. *E. coli* BL21 cells were transformed with the plasmid construct and the expressed MBP–Apoptin was purified by double passage over an amylose column and eluted with 10 mM maltose. The eluate was loaded to an analytical cation-exchanged column, washed, eluted with a linear gradient of NaCl, and protein-containing fractions were dialyzed against phosphate buffered saline (PBS) and concentrated to 40 mg mL⁻¹. Fluorescent MBP–Apoptin was prepared by conjugating Alexa Fluor 647 (AF647) according to manufacturer's instructions (Molecular Probes). Free AF647 was separated from labeled protein by a Bio-Rad BioGel P-30 Fine size-exclusion purification resin.

Polymer Synthesis and Characterization

L-phenylalanine was grafted onto pendant carboxylic acid moieties along the backbone of poly(L-lysine *iso*-phthalamide) to yield PP-75 with a 75% stoichiometric molar addition [21]. In summary, poly(L-lysine *iso*-phthalamide) was prepared by polymerization of aqueous L-lysine methyl ester dihydrochloride with an equivalent amount of *iso*-phthaloyl chloride in acetone and subsequent hydrolysis in dimethyl sulfoxide (DMSO) with ethanolic sodium hydroxide [13–15]. PP-75 was prepared from the coupling reaction for 60 h between poly(L-lysine *iso*-phthalamide) and L-phenylalanine methyl ester hydrochloride/triethylamine (1/2.4, mol mol⁻¹) in an anhydrous DMSO/*N,N'*-dimethylformamide (DMF) binary solvent system by *N,N'*-dicyclohexylcarbodiimide (DCC)/4-dimethylaminopyridine (DMAP) mediated amide coupling chemistry [32,33], followed by hydrolysis of the resulting polymer with ethanolic sodium hydroxide. The solution was precipitated rapidly into five volumes of diethyl ether. The precipitate was collected and redissolved in deionized water. Inorganic salts and residual organic solvents were removed by dialysis against deionized water. The solution was concentrated, titrated to pH 7.4, and then lyophilized to a fine white powder [21]. The structures of poly(L-lysine *iso*-phthalamide) and the grafted PP-75 polymer were confirmed using a Nicolet Nexus FTIR spectrometer (Thermo Fisher Scientific, USA) and a Bruker Advance 500 MHz NMR spectrometer (Bruker Biospin GmbH, Germany). The molecular weight of poly(L-lysine *iso*-phthalamide) ($M_w = 35.7$ kDa, $M_n = 17.9$ kDa, polydispersity = 1.99) was determined using an aqueous gel permeation chromatography (GPC) system (Viscotek, UK), which was calibrated with polyethylene glycol standards. The degree of grafting of PP-75 was measured from its ¹H NMR spectrum, and used to calculate its molecular weight (degree of grafting = 63.2%, $M_n = 24.9$ kDa). The FITC-labeled polymer, PP-75-FITC (1.0 mol% FITC), was prepared by coupling FIT–NH–(CH₂)₂–NH₂, an amino derivative of FITC from the reaction with ethylene diamine using dibutyltin dilaurate as a catalyst, to the carboxylic acid groups of PP-75 using standard DCC/DMAP mediated coupling techniques. PP-75-FITC was purified by dialysis against deionized water and then lyophilized. A low level of labeling was selected here to avoid significant modulation of the polymer properties and fluorescence quenching [20–22].

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Figure 1.

Native PAGE analysis of putative PP-75 MBP-Apoptin association. Polymer and protein were allowed to equilibrate 3 h before running. The long arrow shows MBP-Apoptin band. A) High PP-75 concentration: MBP-Apoptin only ($33 \mu\text{g mL}^{-1}$, $0.6 \mu\text{M}$), $700\text{--}1000 \mu\text{g mL}^{-1}$ PP-75 with and without MBP-Apoptin. B) Low PP-75 concentration: MBP-Apoptin only, $10\text{--}100 \mu\text{g mL}^{-1}$ PP-75 with and without MBP-Apoptin.

Figure 2.

Influence of PP-75-FITC on uptake of MA-AF647 (33 $\mu\text{g mL}^{-1}$, 0.6 μM) by Saos-2 cells. Samples were treated with protein and/or polymer for 1 h and tested after 1 h further incubation. Error bars represent the standard deviation of three samples. A, C) Proportion of MA-AF647 viable + cells. B, D) Mean Alexa Fluor 647 fluorescence of viable cell population. Viable cells were gated based on propidium iodide exclusion.

Figure 3.

Intracellular distribution and colocalization of PP-75-FITC and MA-AF647 in Saos-2 cells. Cells were treated with PP-75-FITC and/or MA-AF647 for 1 h and imaged after further incubation. Fluorescence images collected from photomultiplier tube (PMT)1 are labeled FITC, fluorescence images collected from PMT2 are labeled MA-AF647, and overlay images show FITC and MA-AF647 merged with bright field. A) Cells treated with $100 \mu\text{g mL}^{-1}$ PP-75-FITC at 1 h further incubation. Scale bar: $50 \mu\text{m}$. B) $100 \mu\text{g mL}^{-1}$ PP-75 and $33 \mu\text{g mL}^{-1}$ MA-AF647 at 1 h further incubation. Scale bar: $25 \mu\text{m}$. C) $100 \mu\text{g mL}^{-1}$ PP-75 and $33 \mu\text{g mL}^{-1}$ MA-AF647 at 5 h further incubation showing nuclear localization of MBP-Apoptin.