Drug Delivery

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Phosphatase-Triggered Fusogenic Liposomes for Cytoplasmic Delivery of Cell-Impermeable Compounds**

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Viruses have evolved into what engineers view as sophisticated nanomachines, containing fusion machinery responsible for membrane destabilization and cellular infection.[1–3] Activation of viral fusion proteins exposes fusogenic peptide sequences that bridge the viral and cellular membranes to induce membrane fusion. Enzyme-triggered liposomes that can mimic viral activation might be useful vehicles for delivering proteins or genetic material into cells.[4–6] However for liposomes to stably display fusogenic peptides on their surface, the challenge of incorporating the peptide on the bilayer surface while preventing peptide insertion into the membrane (an event that would compromise bilayer integrity) must be overcome.[7]

Herein, we controlled the membrane-destabilizing activities of fusion peptides by strategically placing phosphate groups within the peptide sequence. We then designed phosphatase-triggered lipid-based particles, termed PTPs, by displaying phosphopeptides on the surface of liposomes. Phosphatases can remove the phosphate groups of the peptides, which then activate membrane fusion between the liposome and another membrane. In cells, this results in cytosolic delivery of the liposome-encapsulated cell-impermeable compounds.

Phosphatases are enzymes responsible for catalyzing the hydrolysis of phosphate esters and can work on a variety of phosphorylated proteins, peptides, nucleotides, alkaloids, phospholipids, and lipopolysaccharides.[8–13] Interestingly, phosphatases are overexpressed in a number of inflammatory and chronic disorders,[14–16] especially in tumor microenvironments.[17,18] The broad range of phosphatases in the body and their selective over-expression in diseased tissue make them an attractive enzymatic trigger for drug carriers.[13,19] As a result, phosphatases have been used for decades as a trigger mechanism for the activation of prodrugs whose water solubility is increased by the attachment of a phosphate.[20,21]

We selected the well-characterized HIV gp41 N-terminus fusion peptide[7,22–24] as a template for designing activable fusogenic phosphopeptides. Phosphate groups were placed in nonconserved positions at the N and C termini[25–27] of a 22 residue peptide to disrupt membrane insertion and increase the polarity of the peptide. Phosphorylated fusion peptides (FP-2PT) exhibited significant control over the membrane-destabilizing properties of fusion peptides (Figure 1). The hydrophilic phosphate groups masked the hydrophobic character of the peptides and inactivated their fusogenic properties. Phosphatase-catalyzed dephosphorylation (FP-2PT-AP) regenerated the membrane-destabilizing properties of the peptides, restoring their fusogenic activity. Activation was phosphatase dependent because heat-inactivated phosphatase or the presence of a phosphatase inhibitor blocked lipid mixing (Figure 1 A). The phosphorylated fusion peptides displayed switch-like behavior: remaining essentially unstructured while phosphorylated but flipping into an alpha helix upon removal of the phosphate groups from the peptide (Figure 1 B). Thus, these phophopeptides have low membrane-destabilizing properties in the absence of phosphatases and high fusogenic activity in the presence of phosphatases.

A lipidated phosphorylated fusion peptide, FP-2PT-Chems, was prepared by conjugation of NHS-Chems (*N*-hydroxysuccinimide cholesteryl hemisuccinate) to a C terminal lysine. The lipidated peptide was then included in the lipid mixture used to prepare PTP liposomes. The sonication and extrusion process produced consistent PTPs with an average hydrodynamic diameter of 100 nm (94.1 ± 6.7 nm), and an average polydispersity index (PDI) of 0.233 (Figure 2 A). No significant change in particle diameter was detected upon increasing the surface density of lipopeptides. Vesicle formation was not possible when non-phosphorylated fusogenic lipopeptides were used, which suggests that the phosphate groups prevent the lipopeptide from inserting into the PTP membrane.

PTPs containing 2 mol% FP-2PT-Chems had a zeta potential of -17 ± 3 mV at physiological pH, whereas liposomes prepared from anionic lipids with a phosphate in the headgroup such as cholesterol phosphates,[13] phosphatidylglycerol (POPG), or inverse phosphocholine (DOCP), have zeta potentials lower than -60 mV (Figure 2 b).[28] Decreasing the FP-2PT-Chems density to 0.5 mol% resulted in a neutral zeta potential. Thus, changes in the surface density of the lipopeptide correlated with changes in zeta potential. Incubation of PTPs containing 2 mol% FP-2PT-Chems with alkaline phosphatase resulted in a zeta potential of $-7.5 \pm$

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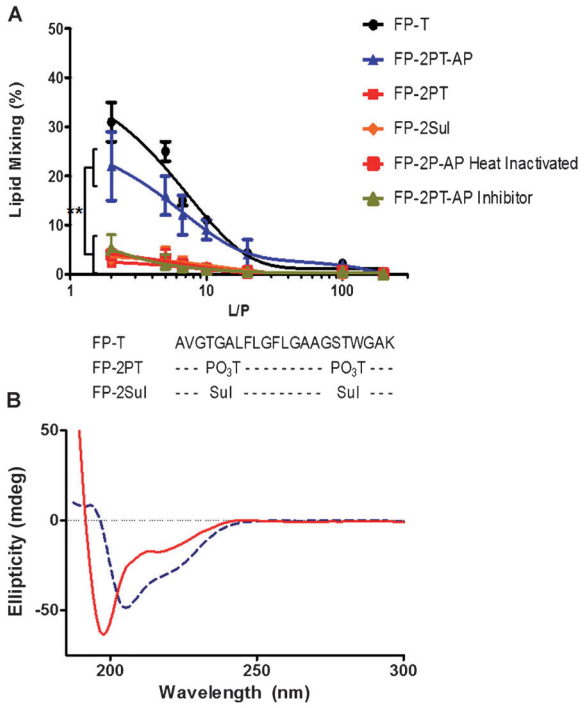

Figure 1. Fusogenic activity and structural changes of phosphorylated fusion peptides. A) The fusogenic activity of fusion peptides (FP-T) was monitored with a lipid mixing assay in the presence and absence of alkaline phosphatase (AP). The sequences of the modified GP41 fusion peptides used in the assays are shown below the graph. Limited lipid mixing was observed for the phosphorylated peptide FP-2PT. Addition of alkaline phosphatase triggered the phosphopeptides (FP-2PT-AP) and restored their lipid mixing activity. The addition of a phosphatase inhibitor, sodium orthovanadate, or heat inactivation, significantly reduced the lipid mixing. A peptide with sulfonated residues (FP-2Sul) also showed low lipid mixing. ** $p < 0.01$. B) Phosphorylated fusion peptides (red) had altered helical structures and increased random-coil content, whereas their nonphosphorylated counterpart (blue) exhibited a predominantly alpha helical structure.

1 mV, which indicates that the PTPs are also substrates for the enzyme. A sulfonated lipid-based particle, containing the lipopeptide FP-2Sul-Chems, was used as a control, since the sulfonate group is not a substrate for phosphatases but imbues nanoliposomes with similar surface characteristics as the PTPs (Figure 2A,B).

To determine the kinetics of dephosphorylation of the PTP by the phosphatase, we monitored phosphate release using a highly sensitive phosphate-binding fluorescent protein sensor that measures nanomolar phosphate concentrations.[29] Exposure of PTPs to alkaline phosphatase resulted in rapid dephosphorylation of the phosphopeptides, with $72 \pm 15\%$ of the phosphates cleaved in ten minutes. The enzymatic activity curve is characterized by a brief lag phase followed by a linear phase (Figure 2C). Dephosphorylation plateaus after ten minutes with an estimated 30% of the phosphate groups remaining uncleaved. A number of factors could explain this. The phosphorylated lipopeptide is incorporated into PTPs by sonication and extrusion, rather than by micelle-transfer techniques. As a result, a percentage of the phosphopeptides end up facing the enclosed aqueous compartment and are inaccessible to the phosphatases. Furthermore, as the PTPs are dephosphorylated they undergo fusion with neighboring liposomes, which reduces the fraction of phosphate groups accessible to the phosphatase.

PTPs were designed to destabilize membranes and induce membrane fusion[30] following dephosphorylation by phosphatases (Figure 2D). Phosphatase triggering of membranes with 2 mol% of FP-2PT-Chems induced $30 \pm 10\%$ lipid mixing as compared to 5% lipid mixing for the free peptide (Figure 2D). Increasing the surface density of phosphopeptide to 5 mol% resulted in a further increase in phosphatase-triggered lipid mixing ($50 \pm 15\%$). Thus the extent of lipid mixing can be modulated by the surface density of the phosphopeptides. Phosphatase treatment of liposomes modified with the control peptide FP-2Sul-Chems did not result in increased lipid mixing (Figure 2D).

Content-release assays with liposomes containing self-quenched carboxyfluorescein (CF) were performed to determine the ability of PTPs to induce membrane leakage. Upon dephosphorylation by alkaline phosphatase, PTPs containing 2 mol% FP-2PT-Chems showed a significant release of CF ($60 \pm 10\%$; Supporting Information, Figure S.1). Exposure of PTPs to a more specific Ser/Thr protein phosphatase (PP1) resulted in nearly complete CF release ($85 \pm 10\%$) of POPC liposomes (Figure S.1). The results of the lipid mixing and content release assays confirmed that PTPs have membrane-destabilizing properties that are suitable for a phosphatase-triggered fusogenic liposomal drug carrier.

We assessed the biocompatibility of PTPs by measuring cell viability, using the MTT assay, over a broad range of PTP concentrations (Figure 3C). A metastatic cell line, B16F10, was selected for this assay because of its high phosphatase expression and because it has previously been used to evaluate the cytotoxic effects of phosphate prodrugs.[31–33] No significant difference in cell toxicity, as compared to POPC liposomes, was measured with FP-2Sul-Chems and FP-2PT-Chems liposomes at concentrations relevant for cellular uptake studies (up to 10 μM). Increasing the concentrations by tenfold reduced the cell viability by $30 \pm 5\%$ in cells treated with FP-2PT-Chems liposomes, as compared to a $18 \pm 5\%$ reduction for cells treated with FP-2Sul-Chems and a $12 \pm 5\%$ reduction for POPC liposomes. The lower viability of cells treated with FP-2PT-Chems liposomes might be attributed to the membrane disrupting properties of fusogenic peptides unmasked by cellular phosphatases.

To investigate the extent and rate of cellular association and internalization of PTPs, fluorescently-labeled PTPs, containing 1 mol% rhodamine-labeled-phosphatidylethanolamine (Rho-PE), were incubated with B16F10 cells and the cellular association was monitored over time by fluorescence microscopy. Association was detected within the first hour for PTPs containing 2 mol% FP-2PT-Chems, with a high percentage of PTPs localized to the cell membrane (Figures S.2, S.3). Cellular accumulation and internalization was greatly enhanced at later time points (Figure S.2E,F). Lower uptake and internalization was observed in cells treated with control liposomes containing 2 mol% FP-2Sul-Chems (Figure S.3C,D). This suggests that the anchored phosphopeptide on the PTP was dephosphorylated, which

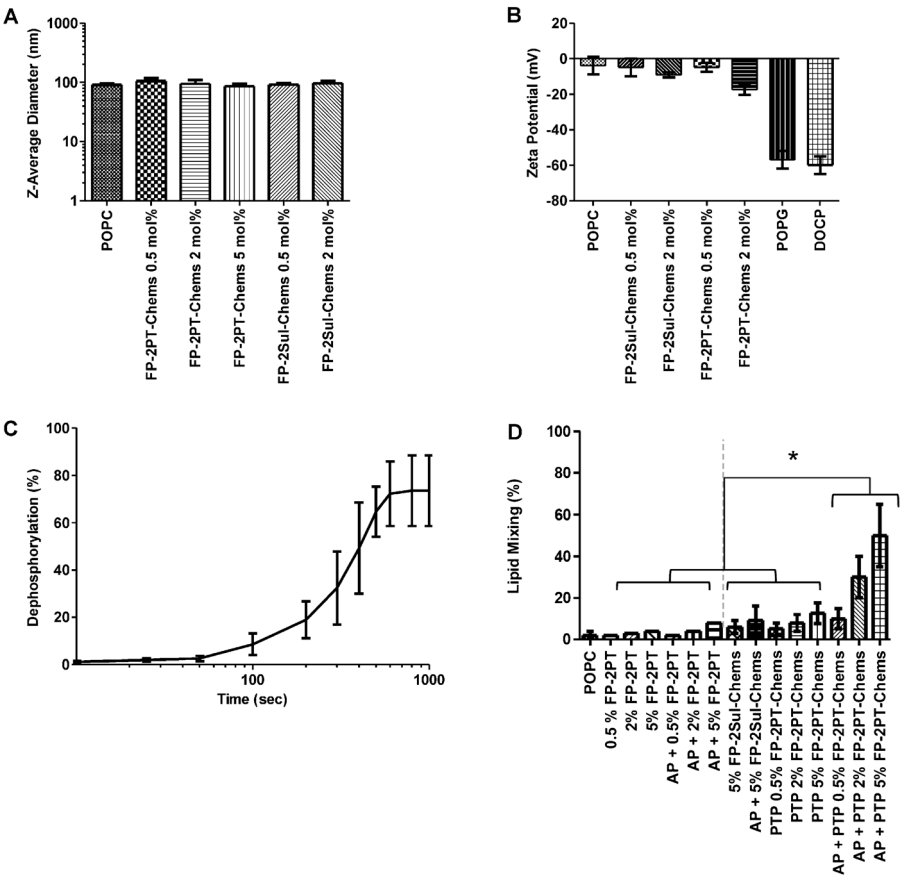

Figure 2. Characterization of A) hydrodynamic diameter, B) zeta potential, C) dephosphorylation, and D) fusogenicity of PTPs. PTPs are on the right side of the dotted line. Significant differences are marked with * asterisk ($p < 0.05$). POPC = 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine.

resulted in greater cellular accumulation. Furthermore, the significant reduction in uptake and accumulation of liposomes containing FP-2Sul-Chems provides further evidence that dephosphorylation is required for PTP internalization.

Flow cytometry of B16F10 cells incubated with fluorescently labeled PTPs confirmed the time-dependent uptake of PTPs (Figure 3 A,B), as well as their enhanced cellular association relative to control liposomes. A gradual increase in fluorescence was measured over time, with a fivefold higher uptake of PTPs compared to control liposomes at six hours. Uptake of liposomes containing FP-2Sul-Chems was at background level with no statistical difference as compared to control liposomes lacking a fusion peptide.

PTPs containing 2 mol% FP-2PT-Chems displayed a threefold increase in uptake as compared to PTP containing 0.5 mol% FP-2PT-Chems (Figure 3 B). Increasing the surface density to 5 mol% did not further increase cellular association, a result confirmed by fluorescence microscopy studies (Figure S.4). The cellular association of control liposomes modified with FP-2Sul-Chems was low (Figure 3 B) and did not increase when the surface density was increased.

Cellular uptake studies were performed in the presence of phosphatase inhibitors[34,35] and under depleted energy conditions to test the hypothesis that active phosphatases are necessary for uptake. Uptake was marginally affected by inhibition of tyrosine phosphatases, but greatly reduced by inhibition of threonine phosphatases. Cells treated with an inhibitor cocktail of both phosphatase inhibitors exhibited no significant uptake (Figure S.5). Incubation of PTP under depleted energy conditions (4 °C, serum-free medium) exhibited significantly reduced uptake and internalization of PTP, implying that uptake is a temperature- and energy-dependent process.[36] These findings support the hypothesis that the uptake of PTP requires active phosphatases that catalyze the dephosphorylation of the fusogenic phosphopeptides.

To be considered a drug carrier, PTPs must be able to encapsulate and retain a cargo and mediate cytosolic delivery. To test this criterion, fluorescent model drugs with different molecular weights were encapsulated. PTPs encapsulating CF, propidium iodide (PPI), or FITC-dextran were prepared by the sonication and extrusion method and purified by size-exclusion chromatography.

PTPs delivered the encapsulated fluorophores to the cytosol of B16F10 cells. Delivery of CF occurred within two hours (Figure 4 A–C). Confocal images indicated cytoplasmic delivery of CF, as well as co-localization of PTPs containing Rho-PE probes near the perinuclear space of B16F10 cells. The delivery of PPI also was time-dependent and accumulated in the perinuclear region of the cell (Figure 4 D–F). When PPI is delivered into the cytosol, it diffuses through the cytosol and into the nucleus where it intercalates with DNA. This leads to a 20–30-fold increase in fluorescence that can be used as a surrogate marker for cytosolic delivery. B16F10 cells incubated with PTPs encapsulating PPI exhibited nuclear fluorescence within four hours, indicating that PPI was released into the cytosol (Figure 4 D). In some instances, the cells exhibited a diffuse red fluorescence throughout the cytoplasm, which occurs when PPI complexes with RNA in the cytosol. Cytosolic delivery of macromolecules also occurred (Figure 4 G–I). When FITC-dextran was delivered to the cytosol, a diffuse fluorescence was observed throughout the cytoplasm of the cell. This fluorescence is easily differentiated from the staining of membranes or intracellular compartments such as vacuoles.[37] Incubation of PTPs encapsulating FITC-dextran demonstrated cytosolic delivery within four hours. Significant particle accumulation around the perinuclear space was also observed (Figure 4 G,I). However, compared to low molecular weight fluorophores, the degree of delivery was reduced at 4 h (Figure 4 H), even

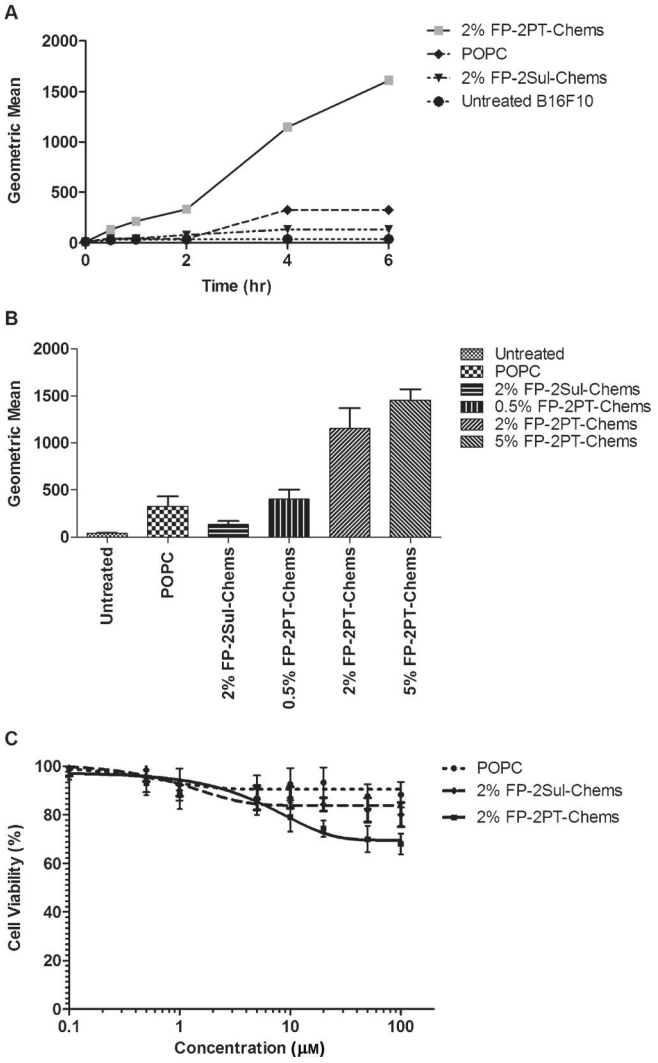

Figure 3. Quantification of cellular association and toxicity of PTPs in cultured cells. A) Flow cytometry measurement of the cellular association of PTPs and controls over time. B) Quantitation of cellular association of liposomes at 4 h. C) Viability of B16F10 cells after treatment with PTPs and liposomes as determined by MTT assay.

though significant PTP accumulation was observed in all cells. Liposomes modified with the sulfonated lipopeptide FP-2Sul-Chems did not mediate cytosolic delivery of the fluorophores (Figure S.6), indicating that removal of the hydrophilic phosphate groups is a requirement for cytosolic delivery.

In summary, phosphorylated lipopeptides on PTPs were substrates for phosphatases, and when dephosphorylated, mediated fusion with the cellular membrane. The membrane-destabilizing potential of PTPs was easily modulated by changing the surface density of the lipopeptide, as well as by the type of phosphatase catalyzing the dephosphorylation. The PTP liposomes associated with cells in a time-dependent manner and mediated cytosolic delivery of their contents. Thus, substrate masking by hydrophilic phosphate groups can be used as a technique to control the membrane-destabilizing properties of fusion peptides and could potentially be used to modulate cell-penetrating peptide systems.[38,39] Finally, the use of low molar fractions of phosphopeptides enables the

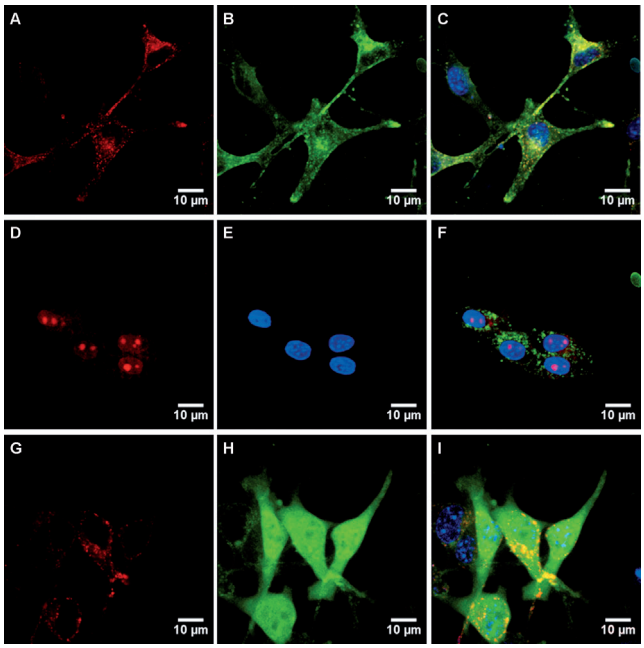

Figure 4. Cytosolic delivery of low molecular-weight fluorophores and fluorescent macromolecules in cultured cells by PTPs modified with FP-2PT-Chems. Representative confocal images show uptake of PTPs and cytoplasmic delivery of a small molecule (CF), intercalating agent (PPI), or macromolecule (FITC-dextran). B16F10 cells were incubated for 2 h with PTPs encapsulating 100 mM CF (A–C), 1 mM PPI (D–F), or 4 h with 1 mM FITC-dextran PTPs (10 kDa; G–I). PTP formulations contained fluorescent lipids Rho-PE (A, G) or NBD-PE (F). Fluorescence of the cargo molecules is shown in B) CF, D) PPI, and H) FITC-dextran. DAPI was used to stain the nuclei of the cells (C, E, F, I).

design of phosphatase-triggered liposomes with reduced negative surface charge. This circumvents the divalent-cation-induced aggregation that plagued earlier phosphatase-triggered liposomes.[13] The potential of PTPs to mediate cytosolic release of cell impermeable molecules, proteins, and nucleic acids is apparent and could begin a new generation of controlled-release therapeutics against cancer and other disorders in which phosphatases are over expressed.

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