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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 the fusion proteins exposes fusogenic peptide sequences that bridge the viral and cellular membranes to induce membrane fusion. Enzymetriggerable liposomes that can mimic viral-like activation might be useful delivery vehicles for proteins and genetic material.⁴⁻⁶ However for liposomes to stably display fusogenic peptides on their surface, one must overcome the challenge of incorporating the peptide on the bilayer surface while preventing peptide insertion into the membrane, an event that would compromise bilayer integrity.⁷

In this report, we gained control over the membrane destabilizing activities of fusion peptides by strategically placing phosphate groups within the peptide sequence. We then engineered phosphatase-triggerable lipid-based particles, termed PTP, 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 the 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 disorders¹⁴⁻¹⁶, 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 engineered carriers.^{13, 19} As a result phosphatases have been used for decades as a trigger mechanism for the activation of pro-drugs whose water solubility is increased by the attachment of a phosphate.^{20, 21}

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We selected the well characterized HIV gp41 N-terminus fusion peptide^{7, 22-24} as a template for designing triggerable fusogenic phosphopeptides. Phosphate groups were placed in non-conserved positions at the N and C termini²⁵⁻²⁷ of a 22 residue peptide in order to disrupt membrane insertion and increase sequence polarity and trigger control. Phosphorylated fusion peptides exhibited significant trigger 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 regenerated the membrane destabilizing properties of the peptides, restoring their fusogenic activity. Triggering was phosphatase dependent since heat-inactivated phosphatase or the presence of a phosphatase inhibitor blocked the lipid mixing (Figure 1a). The phosphorylated fusion peptides displayed switch-like behavior; being essentially unstructured when phosphorylated but flipped into an alpha helix when the phosphate is clipped from the peptide (Figure 1b). Thus, they have low membrane destabilizing properties in the absence of phosphatases, but 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 PTP with an average hydrodynamic diameter of 100 nm (94.1 ± 6.7 nm), and an average PDI of 0.233 (Figure 2a). No significant change in particle diameter was observed by increasing the surface density of lipopeptides. Vesicle formation was not possible when unphosphorylated fusogenic lipopeptides were used, which suggests that the phosphate groups prevent the lipopeptide from inserting into the PTP membrane.

PTP 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 (17), phosphatidylglycerol (POPG), or inverse phosphocholine (DOCP), have zeta potentials lower than -60 mV (Figure 2b).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 PTP containing 2 mol% FP-2PT-Chems with alkaline phosphatase resulted in the PTP having a zeta potential of -7.5 ± 1 mV, which indicates that PTP are also substrates for the enzyme. A sulfonated PTP, containing the lipopeptide FP-2Sul-Chems, was devised as a control peptide sequence since the sulfonate group is not a substrate for phosphatases but imbues nanoliposomes with similar surface characteristics as the PTP (Figure 2a-b).

To determine the kinetics of dephosphorylation of the PTP by phosphatase, we monitored phosphate release using a highly sensitive phosphate-binding fluorescent protein sensor that measures nanomolar phosphate concentrations.²⁹ Exposure of PTP to alkaline phosphatase resulted in rapid dephosphorylation of the phosphopeptides, with $72 \pm 15\%$ of the phosphates cleaved in 10 minutes. The enzymatic progress curve is characterized by a brief lag phase followed by a linear phase (Figure 2c). Dephosphorylation plateaus after 10 minutes with an estimated 30% of the phosphate groups remaining uncleaved. A number of factors can explain this. The phosphorylated lipopeptide is incorporated into PTP 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 PTP are dephosphorylated they undergo fusion with neighboring liposomes, which reduces the fraction of phosphate groups accessible to the phosphatase.

PTP were designed to destabilize membranes and induce membrane fusion³⁰ upon 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 phosphate 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 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 PTP to induce membrane leakage. Upon dephosphorylation by alkaline phosphatase, PTP containing 2 mol% FP-2PT-Chems induced significant CF release ($60\pm 10\%$) (Figure S.1). Exposure of PTP to a more specific ser/thr protein phosphatases (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 PTP have membrane destabilizing properties that are suitable for a phosphatase triggered fusogenic liposomal drug carrier.

We measured the biocompatibility of PTP using the MTT assays over a broad range of PTP lipid concentrations (Figure 3c). The metastatic B16F10 cell line was selected for the assay because of its phosphatase expression and its previous use to evaluate the cytotoxic effects of phosphate pro-drugs.³¹⁻³³ No significant difference in cell toxicity, as compared to POPC, was observed with FP-2Sul-Chems and FP-2PT-Chems liposomes at concentrations relevant for cellular uptake studies ($10\text{ }\mu\text{M}$). Increasing the concentrations by 10-fold reduced the cell viability by $30\pm 5\%$ in cells treated with FP-2PT-Chems liposomes as compared to $18\pm 5\%$ reduction for cells treated with FP-2Sul-Chems and $12\pm 5\%$ 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 phosphatase.

To investigate the extent and time course of cellular association and internalization of PTP, fluorescently-labeled PTP, containing 1 mol% Rho-PE, were incubated with B16F10 cells and the cell association was monitored over time by fluorescence microscopy. Cellular association was observed within the first hour for PTP containing 2 mol% FP-2PT-Chems, with a high percentage of PTP localized to the cell membrane (Figure S.2 and 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 controlled liposomes containing 2 mol% FP-2Sul-Chems (Figure S.3c-d). This suggests that the anchored phosphopeptide on the PTP was dephosphorylated, which 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 a requirement for PTP internalization.

Flow cytometry of B16F10 cells incubated with fluorescently labeled PTP confirmed the time-dependent uptake of PTP (Figure 3a-b), as well as their enhanced cellular association relative to control liposomes. A gradual increase in fluorescence was observed over time, with a 5-fold higher uptake of PTP compared to control liposomes at 6 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.

PTP containing 2 mol% FP-2PT-Chems displayed a 3-fold increase in uptake as compared to PTP containing 0.2 mol% FP-2PT-Chems (Figure 3b). Increasing the surface density to 5 mol% did not further increase cell association, a result confirmed by fluorescence

microscopy studies (Figure S.4). The cell association of control liposomes modified with FP-2Sul-Chems was low and did not increase when the surface density was increased (Figure 3b).

Uptake studies were performed in the presence of phosphatase inhibitors^{34, 35} and under energy depletion 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 energy depletion 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, PTP 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. PTP encapsulating CF, propidium iodide (PPI), or FITC-dextran were prepared by sonication and extrusion techniques and purified by size-exclusion chromatography.

PTP delivered the encapsulated fluorophores to the cytosol of B16F10 cells. Delivery of CF occurred within 2 hours (Figure 4a-c). Confocal images indicated cytoplasmic delivery of CF, as well as co-localization of PTP 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 4d-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 PTP encapsulating PPI exhibited nuclear fluorescence within 4 hours, indicating that PPI was introduced into the cytosol (Figure 4d). In some instances, the cells exhibited a diffused red fluorescence throughout the cytoplasm, which occurs when PPI complexes with RNA in the cytosol. Cytosolic delivery of macromolecules also occurred (Figure 4g-i). When FITC-dextran is delivered to the cytosol, a diffuse fluorescence is observed throughout the cytoplasm of the cell. The fluorescence is easily differentiated from the staining of membranes or intracellular compartments such as vacuoles.³⁷ Incubation of PTP encapsulating FITC-dextran demonstrated cytosolic delivery within 4 hours. Significant particle accumulation around the perinuclear space was also observed (Figure 4g-i). However, compared to low molecular weight fluorophores, the degree of delivery was reduced at 4 hours (Figure 4h), even 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 PTP continued to be active substrates for phosphatases, and when dephosphorylated, mediated membrane fusion. The membrane destabilizing potential of PTP was readily 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 for controlling 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 as trigger moieties enables the design of phosphatase triggerable liposomes with reduced negative surface charge. This circumvents

the divalent cation-induced aggregation effect that plagued earlier phosphatase triggerable liposomes.¹³ The potential of PTP to mediate cytosolic release of cell impermeable molecules, proteins, and nucleic acid is apparent and could lead the way for a new generation of triggerable therapeutics against cancer and other disorders in which phosphatases are over expressed.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Fusogenic Activity and Structural Changes of Phosphorylated Fusion Peptides

A) The fusogenic activity of fusion peptides was monitored with a lipid mixing assay in the presence and absence of alkaline phosphatase. Lipid mixing was dependent on the concentration of fusion peptide or phosphopeptide. An increase in the concentration of the fusion peptide FP-T (black) led to an increase in lipid mixing over a broad range of concentrations. Limited lipid mixing was observed for the phosphopeptide FP-2PT (red). Addition of alkaline phosphatase triggered the phosphopeptides (blue) and restored their lipid mixing activity. The addition of phosphatase inhibitors, such as sodium orthovanadate (dark red), or heat treatment (orange), significantly reduced the phosphatase triggering. Significant differences are marked with ** asterisks ($p < 0.01$). The sequences of the modified GP41 fusion peptides used in the assays are presented under the figure. B) Phosphorylated fusion peptides (red) had altered helical structures and increased random coil content, whereas their unphosphorylated counterpart (blue) exhibited a predominant alpha helical structure. The differences in peptide conformations are representative of a “clip-flip” transition.

Figure 2. Characterization of Hydrodynamic Diameter, Zeta Potential, Dephosphorylation, and Fusogenicity of PTP

A) PTP had on average a 100 nm particle diameter. B) A negative surface charge is observed for most PTP formulations, and is correlated to the phosphopeptide surface density. C) The dephosphorylation of phosphorylated lipopeptides displayed on PTP proceeds through a lag phase followed by a linear phase. D) PTP containing FP-2PT-Chems exhibited enhanced triggered lipid mixing in the presence of phosphatase as compared to untriggered PTP and fusion peptides. PTPs are on the left side of the dotted line. Significant differences are marked with * asterisk ($p < 0.05$).

Figure 3. Quantification of Cell Association and Cell Toxicity of PTP in Cultured Cells

A) Flow cytometry showed that PTP with 2 mol% FP-2PT-Chems liposomes had greater time-dependent uptake than peptide-free liposomes or liposomes with 2 mol% FP-2Sul-Chems. B) At 4 hours, PTP containing FP-2PT-Chems exhibited increased cell association relative to control liposomes. Significant differences are marked with *** asterisks (p-value<0.001). Increasing the surface density of FP-2PT-Chems from 0.5% to 5% resulted in higher cellular association of PTP. C) FP-2PT-Chems PTP exhibited concentration dependent cytotoxicity as revealed by MTT assay.

Figure 4. Cytosolic Delivery of Low-Molecular Weight Fluorophores and Fluorescent Macromolecules in Cultured Cells by PTP Modified with FP-2PT-Chems

Representative confocal images show uptake of PTP and cytoplasmic delivery of small molecules (carboxyfluorescein), intercalating agents (propidium iodide) and macromolecules (FITC-Dextran). (A-C) B16F10 cells were incubated for 2 hours with PTP encapsulating 100 mM of carboxyfluorescein. (D-F) B16F10 cells were incubated for 2 hours with PTP encapsulating 1 mM of propidium iodide. (G-I) B16F10 cells were incubated for 4 hours with PTP encapsulating 1 mM FITC-Dextran (10kd). PTP formulations were prepared with 2 mol% FP-2PT-Chems and contained the fluorescent lipids Rho-PE (A, G) or NBD-PE (F). DAPI was used to stain the nuclei of the cells (C, E, F, I).