

Discovery of a Macropinocytosis-Inducing Peptide Potentiated by Medium-Mediated Intramolecular Disulfide Formation

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Abstract: Macropinocytosis is among ubiquitous cellular uptake mechanisms of peptide-based intracellular delivery. Due to its capability of engulfing large macromolecules, macropinocytosis shows promise as a route for the intracellular uptake of biomacromolecules and nanoparticles. We previously reported SN21, a peptide derived from the N-terminus of stromal cell-derived growth factor 1 α (SDF-1 α), as a potent macropinocytosis inducer. In this work, we obtained the 8-residue analog P4A bearing higher macropinocytosis induction ability. P4A contains vital cysteine residues in its sequence, which immediately reacts with cystine in culture medium to convert into its oxidized forms, including the intramolecularly oxidized form (oxP4A) as the dominant and active species. The conjugate of oxP4A with membrane lytic peptide LK15 delivered bioactive proteins into cells; notably, this peptide delivered functional proteins fused with a negatively charged protein tag at a significantly reduced amount (up to nanomolar range) without compromising the delivery efficiency and the cellular activities of delivered proteins.

Introduction

Access of biomacromolecules into the cell interior enables analysis and modulation of the dynamic intracellular molecular processes, deepening our understanding of life and improving biopharmaceutical application towards intracellular targeting.^[1] However, intracellular delivery remains a challenge due to the molecular size and inherent hydrophilicity of cargo biomacromolecules.^[2a–c]

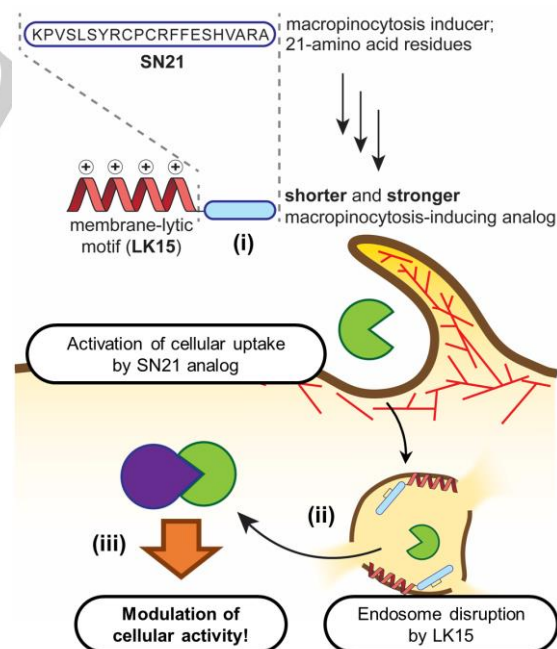


Figure 1. Aims of this study: (i) to establish shorter and more potent analogs of SN21 that activate endocytic (macropinocytic) uptake of functional extracellular cargoes; (ii) to facilitate the endosomal escape of cargoes by conjugation with the endosomolytic peptide LK15; and (iii) to modulate cellular activities.

Numerous cellular delivery strategies employ endocytic pathways for cellular entry, including macropinocytosis, clathrin- and caveolae-mediated endocytosis.^[3] Macropinocytosis is a

type of endocytic pathway, which is considered as stimuli-induced, actin-driven fluid-phase endocytosis, accompanied by membrane ruffling and large-scale engulfment of exogenous fluids and materials into cells.^[4a,b] The resulting endosomes from macropinocytosis, termed macropinosomes, are often reported to exceed 1 μm in diameter, while those of clathrin- and caveolae-mediated endocytosis are typically in a range of around 100 nm.^[5a,b] Macropinocytosis can thus be a preferred route for delivering large macromolecules and nanoparticles into cells.^[4b,6] Reports suggest that membrane ruffling and macropinocytosis are activated by the cell-surface interaction of cell-penetrating peptides (CPPs) and stapled peptides, leading to their facilitated cellular uptake.^[7a-d] Identifying and understanding the mechanism of macropinocytosis, and its inducing agents, should benefit future strategies for intracellular delivery.

Our group reported the potent macropinocytosis induction ability of stromal cell-derived growth factor 1 α (SDF-1 α) through interactions with the CXC-chemokine receptor type 4 (CXCR4).^[8] Following this, we reported a macropinocytosis-inducing peptide SN21, derived from positions 1–21 of SDF-1 α .^[9] By analyzing the cellular uptake of 70 kDa dextran (Dex70; a representative macropinosome marker) and live-cell imaging of actin reorganization, we confirmed the macropinocytosis induction by SN21. Notably, SN21 induced Dex70 uptake with comparable efficacy to that of SDF-1 α , although a 50-fold higher peptide concentration was required. When fused with LK15, a cationic amphiphilic peptide having an endosomolytic activity (SN21-LK15), SN21-LK15 successfully delivered nucleic acids (e.g., siRNA), antibodies, and genome-editing proteins. These results support the intracellular delivery strategy of combining a macropinocytosis inducer with an endosomolytic peptide. SN21-LK15 is composed of 38 amino acid residues; shorter and more potent analogs should show benefits for future practical applications.

This work aimed to obtain shorter, more potent analogs of SN21 to induce macropinocytosis (Fig. 1). SN21 contains two cysteine residues within its sequence. Substituting cysteines in SN21 to alanines revealed the critical importance of these cysteines on macropinocytosis induction of the peptide. Succeeding alanine scanning and amino acid substitution yielded an 8-residue peptide P4A, which is more potent in inducing macropinocytosis compared with SN21. P4A retains the cysteines from SN21. We found that P4A added into culture media immediately formed its intramolecularly disulfide crosslinked form (oxP4A) and the dimers, where oxP4A formed dominantly. This transformation is attributed to the cysteines and cysteines contained within the medium. Finally, oxP4A conjugated with LK15 effectively delivered proteins into cells. This combination shows a particular benefit for delivering proteins bearing a negatively charged protein tag; efficient delivery remained even at a significantly reduced amount of functional proteins fused with supernegatively charged green fluorescent protein.

Results and Discussion

SN21 requires cysteine residues to induce macropinocytosis

As described above, the sequence of SN21 corresponds to the N-terminal 21-residues of SDF-1 α (Fig. 2A). SDF-1 α contains two intramolecular disulfide linkages (Cys9-Cys34 and Cys11-Cys50).^[10] The Cys9 and Cys11 are retained in the SN21 sequence but not their counterpart cysteines. To design shorter and more potent SN21 macropinocytosis-inducing analogs, substituting Cys9 and Cys11 should be practical to avoid possible cysteine modifications (e.g., oxidation and alkylation), making handling easier. We synthesized SN21 mutants with cysteine-to-alanine (C-to-A) substitutions on Cys9 or Cys11 (C9A, C11A, and C9A/C11A in Fig. 2A) and analyzed their ability to induce macropinocytosis. Increased cellular uptake of 70 kDa dextran is a gold standard for assessing macropinocytosis induction; 70 kDa dextran is a fluid-phase endocytosis marker that is difficult to be taken up by endocytosis pathways having smaller-sized endosomes due to the size limitation (i.e., clathrin-mediated and caveolae-mediated endocytosis).^[11] HeLa cells treated with fluorescein-labeled 70kDa dextran (Dex70-FL) in the presence of SN21 for 30 min yielded a marked increase in dextran uptake of ~80% (Fig. 2B).

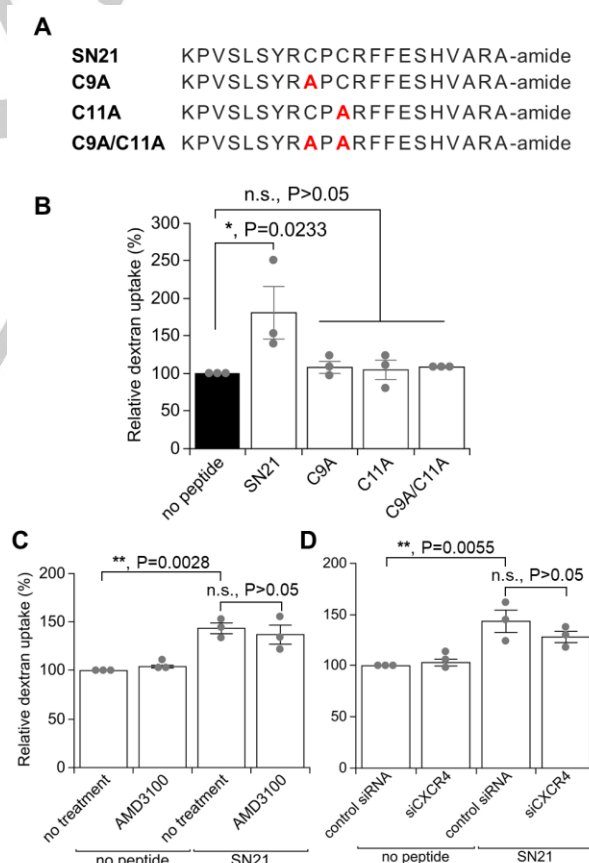


Figure 2. A) Primary sequences of SN21 and C-to-A mutants C9A, C11A, and C9A/C11A. B) Dex70-FL uptake induced in serum-starved HeLa cell treated with 5 μM peptides and 0.5 mg/mL Dex70-FL in α -MEM for 30 min. Dex70-FL uptake in serum-starved cells in the presence of C) CXCR4 antagonist AMD3100 (5 μM), and in D) CXCR4-knockdown HeLa cells. Data are presented as mean $\pm$ standard error (SE) of three independent experiments (n = 3). One-way Analysis of Variance (ANOVA) followed by (B) Dunnett's post-hoc analysis, and (C, D) Tukey's post-hoc analysis (*, $P < 0.05$; **, $P < 0.01$; n.s., not significant).

Unexpectedly, the C-to-A mutation of either (or both) cysteines completely abolished this increase, suggesting the indispensable role of these cysteines for macropinocytosis induction by SN21. Additionally, CXCR4 antagonist AMD3100 treatment, and siRNA knockdown of CXCR4 yielded no significant decrease of Dex70-FL uptake by SN21 (Fig. 2C, D and Fig. S1), suggesting no significant involvement of CXCR4 in SN21-induced macropinocytosis. A nanoluciferase-based bioluminescence resonance energy transfer (NanoBRET) competition assay further confirmed the above results (Fig. S2, details of this analysis is available in the Supporting Information).^[12] These results suggest SN21 employs a CXCR4-independent mode of macropinocytosis induction, distinct from that of the original SDF-1 α , while showing the importance of its cysteine residues.

Establishing short and potent macropinocytosis-inducing analogs of SN21

To further analyze other essential amino acid residues for macropinocytosis induction by SN21, we conducted alanine scanning on SN21. Mutants with alanine substitutions on Tyr7, Arg8, Phe13, and Phe14 showed decreased ability to stimulate Dex70-FL uptake than SN21 (Fig. 3A), suggesting the importance of residues from positions 7 to 14. Further evaluation showed that the peptide corresponding to positions 7 – 14 (sSN21) had a similar activity with SN21. Substituting Pro4 of sSN21 with alanine or glycine (P4A or P4G, respectively) could affect peptide conformation and the modes of interaction with cells. P4A and P4G exhibited dextran uptake of ~170% and ~85% higher than no peptide control, respectively (Fig. 3B, C). Since P4A showed the highest dextran uptake, we used this peptide for the following experiments. Like SN21, macropinocytosis inhibitors [5-(*N*-Ethyl-*N*-isopropyl) amiloride (EIPA)^[13] and wortmannin^[14] decreased the dextran uptake induced by P4A (Fig. 3D). Also, live-cell imaging by confocal laser scanning microscopy (CLSM) of HeLa cells stably expressing F-actin binding peptide lifeact^[15] fused with mCherry (ImHeLa) showed marked lamellipodia (actin-rich membrane protrusions) formation – reflecting actin reorganization and dynamic membrane ruffling associated with macropinocytosis activation (Fig. 3E). These results suggest P4A treatment induces macropinocytosis.

P4A also requires its Cys residues for the induction of macropinocytosis. The C-to-A mutants of P4A (P4A/C3A, P4A/C5A, P4A/C3A/C5A) failed to induce Dex70-FL uptake in treated cells (Fig. 3F, G). To further obtain information on the role of these cysteines, we prepared alkylated and pyridinesulfonylated P4A analogs (alkyl-P4A and Pys-P4A, respectively, Fig. S3A). Alkyl-P4A was prepared by treating P4A with iodoacetamide to block the reactivity of the cysteine thiols.^[16] Pys-P4A was obtained by the treatment with 2,2'-dithiodipyridine where the thiol moieties are activated by pyridinesulfonylation to facilitate interaction with other thiols to form disulfides.^[17a,b] Alkylation of P4A thiols abolished its ability to induce dextran uptake (Fig. S3B, alkyl-P4A), while thiol activation led to further increase in the dextran uptake obtained by P4A (Fig. S3B, Pys-P4A). These data suggest that, although shorter, P4A showed better dextran uptake induction compared with SN21 while requiring the presence of its cysteine residues to induce macropinocytosis.

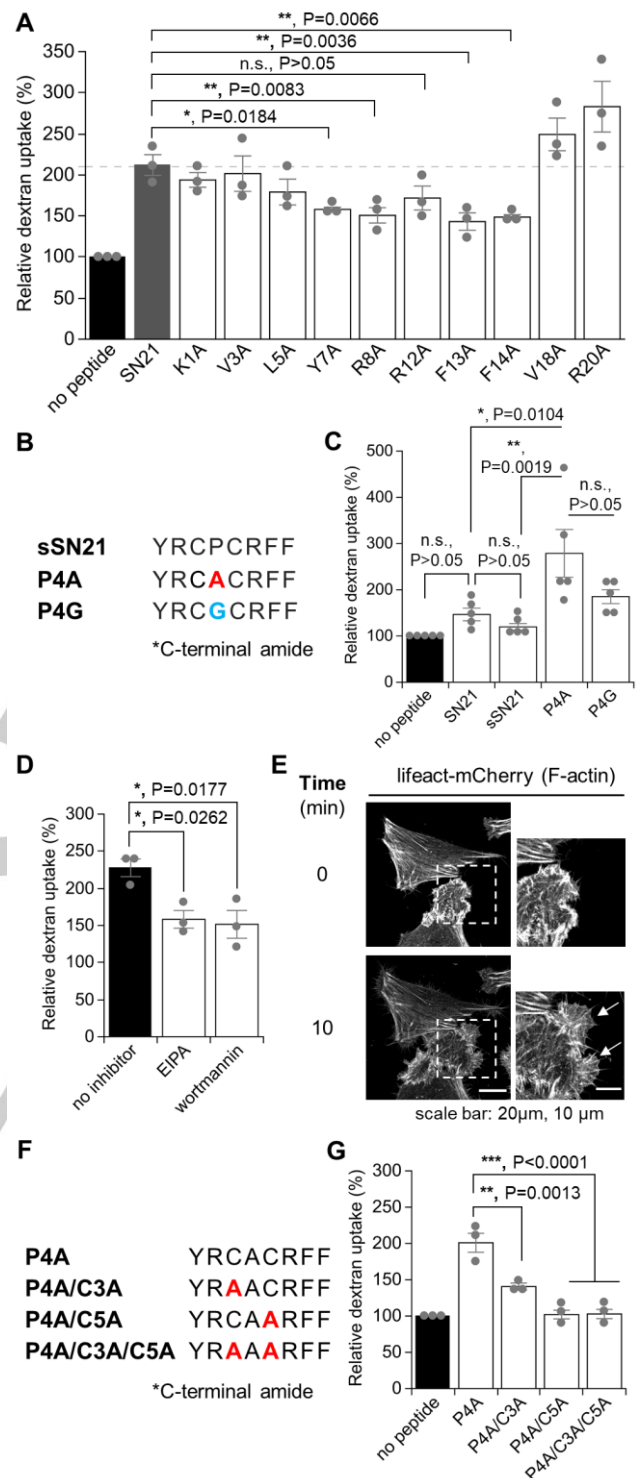


Figure 3. A) Alanine scanning of SN21. Serum-starved HeLa cells were treated with 5 μ M alanine mutants of SN21 in the presence of 0.5 mg/mL Dex70-FL in α -MEM for 30 min. B) Primary sequences of short SN21 (sSN21), P4A, and P4G. C) Induced Dex70-FL uptake in serum-starved HeLa cells treated with 5 μ M peptides and 0.5 mg/mL Dex70-FL in α -MEM for 30 min. D) Dex70-FL uptake induced by P4A in the presence of macropinocytosis inhibitors. Serum-starved cells were pre-treated with 100 μ M EIPA or 500 nM wortmannin in α -MEM for 30 min prior to peptide and Dex70-FL treatment in the presence of inhibitors. E) Formation of lamellipodia (white arrows) after addition of P4A to lifeact-mCherry-expressing HeLa cells (ImHeLa). CLSM images just before (0 min) and 10 min after oxP4A addition are shown. F) C-to-A mutants of P4A and their G) induction of Dex70-FL uptake. Data are presented as mean $\pm$ SE of three independent experiments (n = 3) or five independent experiments (n = 5) for (C). (A) Student's *t*-test, (C, D, and G) one-way ANOVA followed by Tukey's post-hoc analysis (*, *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.001; n.s., not significant).

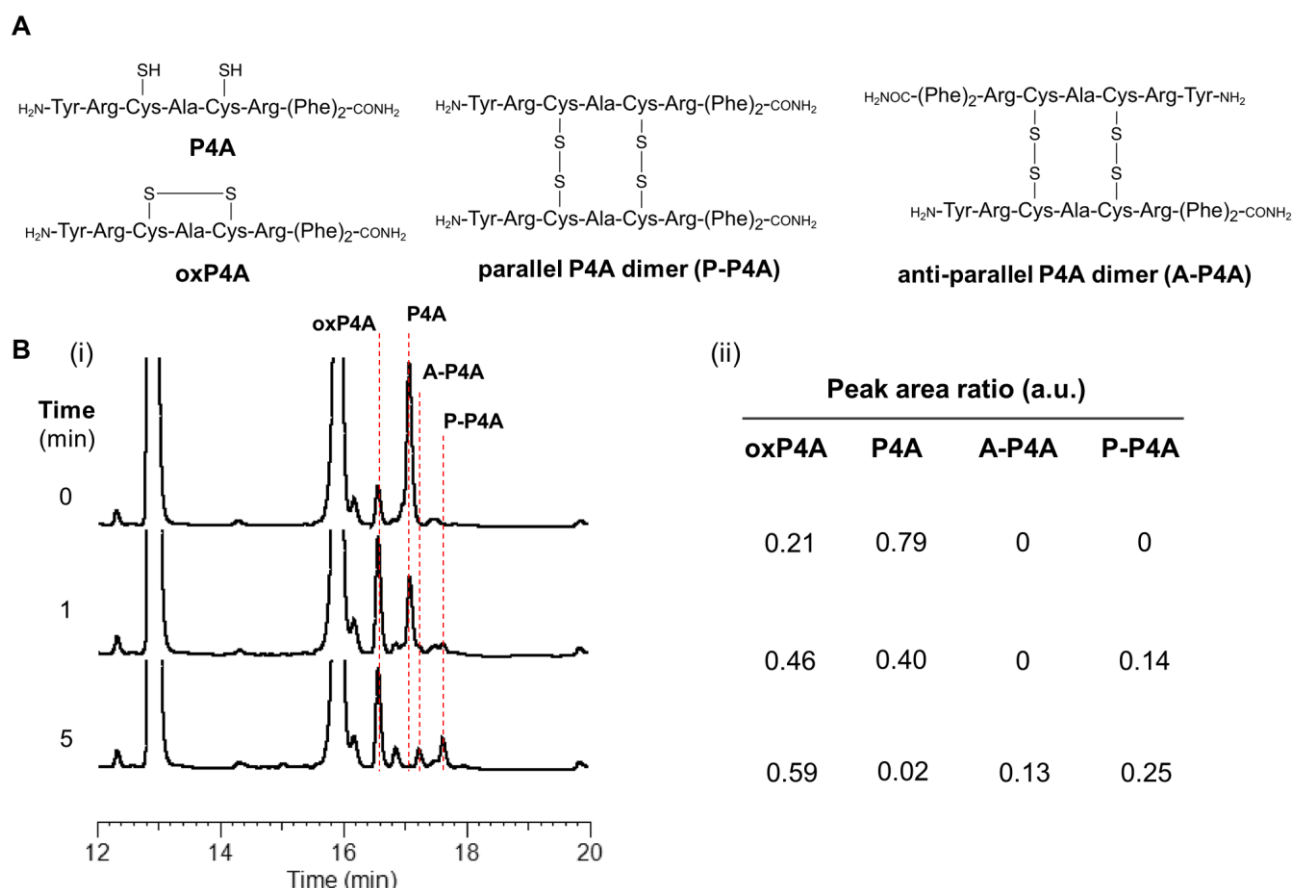


Figure 4. A) Primary structures of potential products of thiol-disulfide exchange reaction by P4A. B) (i) HPLC chromatogram of P4A added to α -MEM, incubated in 37 °C for 0, 1, and 5 min and (ii) corresponding peak area ratios (arbitrary unit, a.u.) of formed oxP4A, A-P4A, and P-P4A [column: COSMOSIL μ C18-ARII (4.5 $\times$ 250 mm, Nacalai Tesque); A = H₂O-0.1% trifluoroacetic acid (TFA), B = acetonitrile-0.1% TFA; gradient: 5 – 55% B, 25 min; flow rate: 1 mL/min; wavelength: 220 nm; temp = 25 °C].

Spontaneous chemical transformation of P4A into more potent oxidized analogs in cystine-containing medium

Recognizing the importance of both cysteines of P4A to induce dextran uptake (Fig. 3), we hypothesized that the reactivity of cysteines on this peptide plays a role in their macropinocytosis induction. The cell culture medium used in this study [α -minimum essential medium (α -MEM), FUJIFILM Wako Pure Chemicals (catalog number 135-15175)] contains high concentrations of L-cysteine and L-cystine (0.5 and 0.1 mM, respectively). P4A may undergo oxidation, producing intramolecular oxidized P4A (oxP4A), and parallel and anti-parallel dimers of P4A (P-P4A and A-P4A, respectively) (Fig. 4A). We confirmed this by adding P4A to α -MEM (final concentration of 5 μ M) and incubating the solution at 37°C. Time-course analysis of the reaction was conducted using high-performance liquid chromatography (HPLC) (Fig. 4B-(i)).^[18] Authentic samples of oxP4A, P-P4A, and A-P4A were prepared to confirm the identity of the products formed in the above time-course analysis (Fig. S4 – S7). The generated peaks were also collected and analyzed for their masses by matrix-assisted laser desorption/ionization mass spectroscopy (MALDI-TOF MS).

We observed a rapid conversion of P4A to the oxidized forms. Immediately after adding P4A into α -MEM, ~20% of P4A converted to oxP4A (Fig. 4B-(i), (ii)). After 1 min, the amount of oxP4A already exceeded that of the original P4A while generating the parallel dimer P-P4A. After 5 min, almost all of P4A converted to other species, where the ratio of formed

oxP4A, anti-parallel dimer A-P4A, and P-P4A was around 5:1:2. Other than these products, there were no prominent observable peaks derived from P4A. Since α -MEM (which contains 0.5 mM L-cysteine and 0.1 mM L-cystine) is assumed to be a redox buffer, oxP4A is considered to be the most preferred product kinetically and thermodynamically, followed by P-P4A and A-P4A.

Given that L-cysteine and L-cystine in α -MEM are potentially responsible for the conversion of P4A, amino acid-free α -MEM [designated as α -MEM (AA–)] was formulated, in which the major components were retained except for all of the amino acids (including L-cysteine and L-cystine) (Table S2). We then analyzed the oxidized peptide formation after P4A addition to α -MEM (AA–) (Fig. 5-(ii)). HPLC analysis showed no significant conversion of P4A when incubated in α -MEM (AA–); P4A remained mostly unchanged, with only a small fraction (less than 10%) forming oxP4A after 30 min of incubation. To study the individual effects of L-cysteine and L-cystine within the media, we conducted the same experiment using α -MEM (AA–) supplemented with L-cysteine (0.5 mM) or L-cystine (0.1 mM). As shown in Fig. 5-(iii), P4A in α -MEM (AA–) containing 0.5 mM L-cysteine remained intact with only minimal formation of oxP4A, similar to α -MEM (AA–) incubation. In striking contrast, the addition of P4A to α -MEM (AA–) containing 0.1 mM L-cystine resulted in a similar conversion pattern as observed in α -MEM addition [Fig. 5-(iv)]. We also observed one or two cysteine molecule adducts on P4A (P4A + 1 Cys and P4A + 2 Cys).

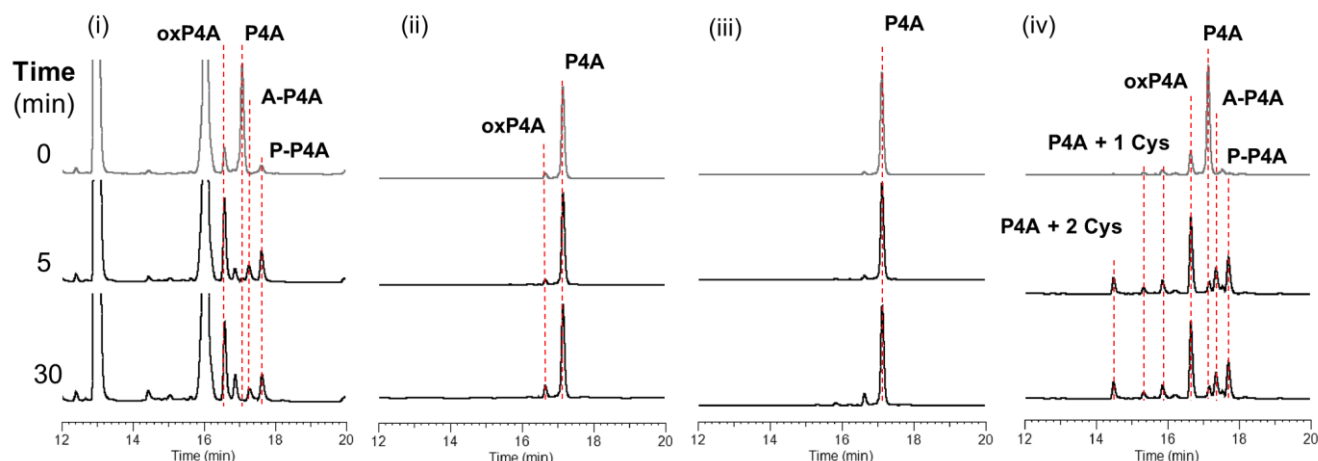


Figure 5. HPLC chromatogram of P4A incubated in (i) α -MEM, (ii) α -MEM (AA⁻), (iii) α -MEM (AA⁻) with 0.5 mM L-cysteine, and (iv) α -MEM (AA⁻) with 0.1 mM L-cysteine at specified incubation times [column: COSMOSIL μ C18-ARII (4.5 $\times$ 250 mm, Nacalai Tesque); A = H₂O-0.1% TFA, B = acetonitrile-0.1% TFA; gradient: 5 – 55% B, 25 min; flow rate: 1 mL/min; wavelength: 220 nm; temp = 25 $^{\circ}$ C].

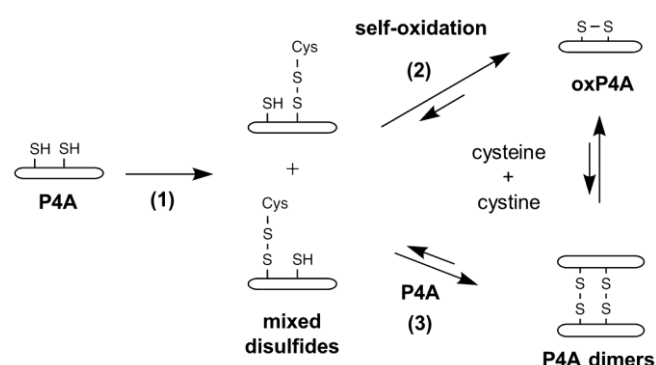


Figure 6. Scheme for hypothesized extracellular thiol-disulfide exchange performed by P4A in α -MEM. (1) P4A reacts with cystine within the media, forming mixed disulfides (P4A + 1 Cys). The reaction may proceed either by (2) intramolecular oxidation, forming oxP4A, or by (3) intermolecular oxidation with another molecule of P4A, forming dimers of P4A.

These are hypothesized intermediates, which could be precursors before oxP4A or P4A dimer formation in α -MEM. These HPLC results also suggest that redox conditions in the presence of L-cysteine and L-cystine facilitate the chemical thiol-disulfide exchange reaction of P4A (Fig. 6).

To understand the thiol-disulfide exchange reactions in more detail, we analyzed the reactions of the oxidized forms of P4A (i.e., oxP4A, P-P4A, and A-P4A) when incubated in α -MEM (Fig. S8A). The HPLC profile obtained by incubating oxP4A in α -MEM yielded oxP4A as the prominent peak together with minor A-P4A and P-P4A peaks after 30 min incubation (Fig. S8A-(i)). On the other hand, incubation of A-P4A and P-P4A yielded oxP4A as a major product, supporting the preferential formation of oxP4A in the redox condition (Fig. S8A-(ii), (iii)). No conversion among the oxidized P4A species was observed in α -MEM (AA⁻) (Fig. S8B) or in α -MEM (AA⁻) with 0.1 mM L-cystine (Fig. S8D).

Alternatively, incubation in α -MEM (AA⁻) with 0.5 mM L-cysteine led to the reduction of the oxidized forms, yielding P4A (reduced form) as the primary product (Fig. S8C). These observations well correlated with the modes of redox exchange of P4A (reduced form) in α -MEM yielding oxP4A as the primary product.

Since P4A added to medium produces its oxidized analogs, we evaluated the individual dextran uptake induction of P4A, oxP4A, P-P4A, and A-P4A. Data showed that A-P4A and P4A had similar induction, while oxP4A and P-P4A yielded higher induction of Dex70 uptake by 55% and 48% higher than P4A, respectively (Fig. S9). We used oxP4A for succeeding experiments because (1) this analog is considered the primary product formed after the thiol exchange reactions, and (2) its simplicity and practicality in both synthesis and usage. Unfortunately, the activity of oxP4A is lost in the presence of serum (Fig. S10). Circular dichroism (CD) spectroscopy suggested no typical secondary structures for both P4A and oxP4A, as in the case of SN21 (Fig. S11). More sophisticated structural analysis techniques must be performed to visualize the acquired structure of oxP4A.

We also evaluated whether SN21 performs the same exchange reaction similar to P4A. Although some fraction of SN21 transformed into its oxidized form (oxSN21) after 5 min, SN21 incompletely converts to oxSN21 even after 30 min (Fig. S12A). Unlike P4A and oxP4A, SN21 and oxSN21 also showed insignificant difference in inducing dextran uptake in HeLa cells (Fig. S12B), suggesting that the ability of SN21 to stimulate dextran uptake is less reliant on its oxidation state. Additionally, C-to-A mutants of SN21 showed similar incomplete conversion to oxidized forms when incubated in media (Fig. S13). These data implies that the oxidation state is not a critical factor for C-to-A mutants (i.e., Y7A, R8A, F13A, and F14A) in affecting the modes of dextran uptake; rather, the decreased activity should be due to the loss of important amino acid residues.

Reports suggest that thiol-disulfide exchange of cysteine-bearing cell-penetrating peptides,^[19a,b] nanoparticles,^[20] and oligonucleotides^[21a,b] with cell surface molecules facilitates direct cellular uptake. Our data shows P4A performs the exchange reaction in the medium prior to interaction with cells, converting into the more potent macropinocytosis inducer oxP4A – suggesting a conceptual difference from previous reports. Additionally, the intramolecular disulfide bond on oxP4A may impart structural rigidity, allowing the peptide to interact and activate cell-surface receptors of macropinocytosis. Taking these into account, work is currently underway to further elucidate the macropinocytosis induction mechanisms of SN21, oxP4A, and P-P4A.

Intracellular delivery of functional proteins using oxP4A conjugated with a membrane-lytic peptide LK15

Conjugating a membrane-lytic peptide as an endosomolytic moiety with macropinocytosis-inducing peptides is a promising strategy for efficient intracellular delivery.^[7d,9] We previously showed that conjugating SN21 with a strong membrane-lytic peptide LK15^[22] (SN21-LK15: KPVSLSYRCPCRFFESHVARA-GG-KLLKLLKLLKLLK-amide) accomplishes effective cytosolic delivery of functional biomacromolecules.^[9] Since oxP4A yielded enhanced macropinocytosis induction, we prepared the conjugates of oxP4A with LK15 connected via a Gly-Gly linker (Fig. 7A). Considering the possibility that oxP4A may have different modes of interaction with cells or cargos from SN21, a conjugate having LK15 on the N-terminal of oxP4A (LK15-oxP4A) was also prepared (Fig. 7A). Protein delivery ability of these conjugates were analyzed using a Cre recombinase-based assay (Fig. 7B).^[23] Briefly, cells transfected with a *loxP-DsRed-loxP-EGFP* reporter plasmid initially express DsRed. Successful delivery of functional Cre results in the recombination of the *loxP* sites – removing the DsRed coding sequence and allowing the expression of enhanced green fluorescent protein (EGFP). The efficacy in delivery was analyzed in terms of the percentages of the cells expressing EGFP among DsRed-expressing cells (i.e., plasmid-transfected cells). The cells were incubated with 2.5 μ M Cre recombinase in the presence of 5 μ M each oxP4A conjugate. A marginal number of cells treated in the presence of oxP4A-LK15 showed EGFP expression (Fig. 7C, oxP4A-LK15), while LK15-oxP4A treatment attained marked expression of EGFP (Fig. 7C, LK15-oxP4A). Cells treated with Cre in the absence of peptide or the presence of 5 μ M oxP4A showed no such recombination (Fig. S14). Thus, we employed LK15-oxP4A for further studies. LK15-oxP4A had a comparable Cre delivery activity with SN21-LK15 as quantified using flow cytometry. The coinubation with 5 μ M LK15-oxP4A and Cre resulted in 39% of cells expressing EGFP (i.e., cells attaining cytosolic delivery of Cre) among DsRed expressing cells, whereas coinubation 2 μ M SN21-LK15 (non-cytotoxic concentration)^[9] resulted in 36% (Fig. 7D, Fig. S15). LK15-oxP4A (5 μ M) showed no significant cytotoxicity when analyzed by WST-8 assay (a mitochondrial dehydrogenase activity-based assay),^[24] while LK15 had considerable cytotoxicity at this concentration (Fig. S16).

Similarly, the delivery efficacy of LK15-oxP4A was further evaluated using EGFP fused with a nuclear localization signal (NLS) (NLS-EGFP) (Fig. 8A). NLS is a tag sequence for cellular proteins to be delivered or localized in the nucleus. For this application, we used the large T-antigen derived NLS of simian

virus 40 (SV40) (PKKKRKV).^[25] HeLa cells treated with 10 μ M NLS-EGFP for 3 h in the presence of 5 μ M LK15-oxP4A yielded 53% of cells having nuclear-localized signals of EGFP (Fig. 8A-(ii), NLS-EGFP). In contrast, SN21-LK15 failed to deliver 10 μ M NLS-EGFP, while yielding a limited number of GFP-positive cells when treated with 50 μ M NLS-EGFP (Fig. S17).

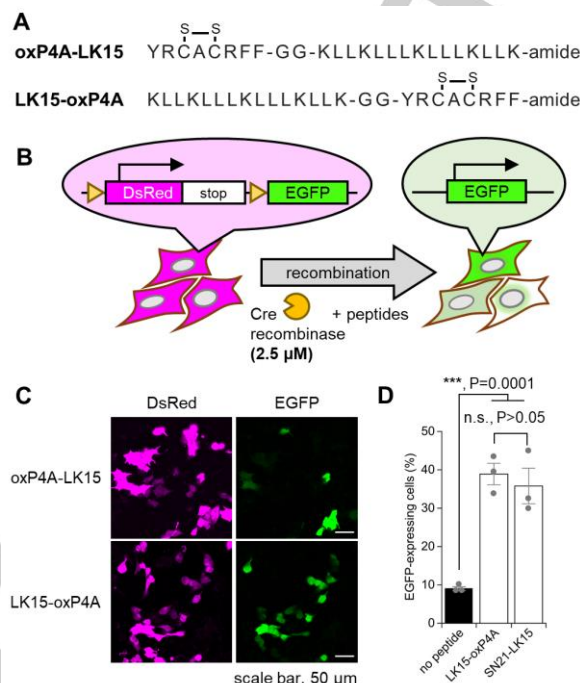


Figure 7. A) Sequences of oxP4A-LK15 and LK15-oxP4A. B) Cre recombination assay. C) Cre delivery using oxP4A-LK15 and LK15-oxP4A. Reporter cells were treated with 2.5 μ M Cre in the presence of 5 μ M oxP4A-LK15 or LK15-oxP4A for 3 h. Images were captured 24 h after cell wash and post-incubation using FV3000 CLSM system. D) Evaluation of EGFP-expressing cells (%) after treatment with 5 μ M LK15-oxP4A or 2 μ M SN21-LK15 as using flow cytometry. Data are presented as mean $\pm$ SE of three independent experiments ($n = 3$). One-way ANOVA followed by Tukey's post-hoc analysis (***, $P < 0.001$; n.s., not significant).

oxP4A showed the applicability for intracellular protein delivery when fused with a membrane-lytic peptide LK15. The successful nuclear localization of NLS-EGFP also exemplifies the feasibility of using LK15-oxP4A for delivering exogenous proteins that targets subcellular compartments. Structural analysis by CD spectroscopy suggested some helical propensity for LK15-oxP4A in aqueous buffer [phosphate buffered saline (PBS), Fig. S11]. Additional structural analysis may be preferred to fully understand the contribution of the structure of LK15-oxP4A to its protein delivery activity.

Enhanced LK15-oxP4A-mediated delivery of functional proteins fused with supernegatively charged protein

LK15 is a cationic amphiphilic peptide, and negatively charged proteins may be preferably delivered into cells by incubation with LK15-oxP4A. The delivery of supercharged GFP bearing -30 negative charges tagged with an NLS [NLS-(-30)GFP] showed this possibility.^[26a,b] Using only 0.5 μ M NLS-(-30)GFP, 68% of cells showed nuclear delivery in the presence of LK15-oxP4A (5 μ M) (Fig. 8A-(ii), NLS-(-30)GFP). The efficacy

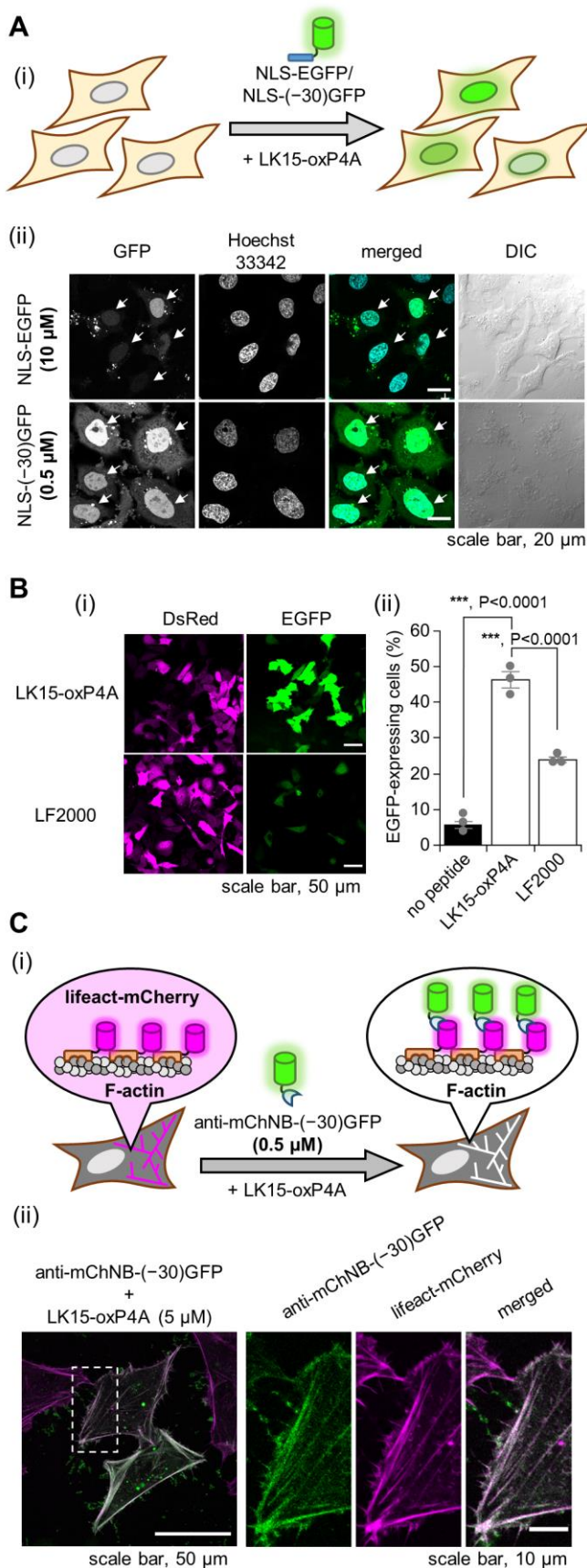


Figure 8. A) (i) Delivery of NLS-EGFP or NLS-(30)GFP. (ii) GFP signal localized within the nucleus (white arrows) in cells treated with 10 μ M NLS-EGFP or 0.5 μ M NLS-(30)GFP with 5 μ M LK15-oxP4A. B) Delivery of 0.5 μ M d(30)GFP-Cre into cells in the presence of 5 μ M LK15-oxP4A or LF2000. (i) EGFP-expressing cells after treatment and (ii) Percentage of EGFP-positive cells after treatment with d(30)GFP-Cre in the presence of LK15-oxP4A or LF2000 analyzed by flow cytometry. Data are presented as mean $\pm$ SE of three independent experiments ($n = 3$). One-way ANOVA followed by Tukey's post-hoc analysis (***, $P < 0.001$). C) (i) Delivery of anti-mChNB-(30)GFP into ImHeLa cells. (ii) ImHeLa cells treated with 5 μ M LK15-oxP4A and 0.5 μ M anti-mChNB-(30)GFP shows colocalization of mCherry and GFP signals along the actin cytoskeleton. The white box indicates a magnified section in the right. Images were captured using the Olympus FV3000 CLSM system.

of this was higher than that attained by using 10 μ M NLS-EGFP (53%; Fig. 8A(ii), NLS-EGFP). Use of 1 μ M LK15 (non-cytotoxic concentration) yielded no substantive nuclear delivery of NLS-(30)GFP (Fig. S18, LK15). A recent study reports a delivery approach using supernegatively charged proteins by coinubation with Lipofectamine 2000 (LF2000).^[27]

However, cells treated with 0.5 μ M NLS-(30)GFP in the presence LF2000 [1 μ L in 200 μ L α -MEM] for 3 h yielded only 32% of cells having nuclear GFP signals (Fig. S18, LF2000). LK15-oxP4A failed to deliver NLS-(30)GFP in 4 $^{\circ}$ C, and a significantly decreased number of cells showed GFP signal within the nucleus in the presence of EIPA and wortmannin (Fig. S19). These data indicate that the activity of LK15-oxP4A relies predominantly on the ability to induce endocytic uptake, particularly macropinocytosis. Transient pore-formation may also contribute to protein delivery, although this method should be highly unlikely or very minimal given the above findings. The Cre recombination assay similarly supported the ability of LK15-oxP4A to deliver functional proteins tagged with the supernegatively charged protein. We engineered Cre recombinase fused with a chromophore denatured (30)GFP [d(30)GFP-Cre] so as not to overlap with the signal from the generated EGFP after gene recombination. Treatment of reporter cells with LK15-oxP4A and 0.5 μ M d(30)GFP-Cre resulted in a similar extent of recombination activity with 2.5 μ M wild-type Cre; 46% of reporter cells showed EGFP expression when treated with 0.5 μ M d(30)GFP-Cre in the presence of 5 μ M LK15-oxP4A (Fig. 8B and Fig. S20). Co-treatment with LF2000 only resulted in 24% of cells expressing EGFP. This strategy effectively lowered the required (30)GFP-fused protein concentration by 1/10 of the wild-type protein concentration without diminished efficacy.

Finally, we exemplified the potential applicability of the above approach to nanobody-based targeting of intracellular proteins (Fig. 8C). Nanobodies (~15 kDa) are the single antigen-binding variable domain (VHH) from heavy chain antibodies naturally produced by camelids.^[28a,b] Due to its size and relatively simple design and production, there is growing interest in the therapeutic potentials of nanobodies.^[29a-c] As describe in Fig. 3E, lifeact-mCherry is an F-actin binding protein. A fusion protein of anti-mCherry nanobody (anti-mChNB)^[30] and (30)GFP [anti-mChNB-(30)GFP] was prepared and applied to ImHeLa (HeLa cells stably expressing lifeact-mCherry). Around 67% of ImHeLa cells treated with 0.5 μ M anti-mChNB-(30)GFP in the presence of 5 μ M LK15-oxP4A showed cells having colocalized mCherry and GFP signals along the actin cytoskeleton (Fig. 8C(ii); Fig. S21). Together, these data illustrate an advantage of the delivery system for reducing the required protein concentrations by employing a negatively-charged protein tag – a desired property for intracellular delivery systems.

Conclusion

This work highlights an 8-residue P4A that significantly accelerates the uptake of exogenous proteins through macropinocytosis. This activity is due to the structural alteration of P4A upon addition to the cell culture medium containing cystine and cysteines, yielding the intramolecularly oxidized form (oxP4A), together with parallel and anti-parallel forms (P-P4A and A-P4A). We derived P4A from the previously reported macropinocytosis-inducing peptide SN21. Although the amino acid sequence of SN21 corresponds to the N-terminal 21 residues of SDF-1 α (an intrinsic ligand of a chemokine receptor CXCR4), CXCR4 inhibition and knockdown did not affect the macropinocytosis-induction ability of the peptide. A nanoBRET-based interaction assay additionally confirmed no significant interaction with CXCR4. Alanine scanning revealed that residues from Tyr7 to Phe14 are essential for the macropinocytosis induction of SN21, wherein Cys9 and Cys11 play a crucial role. Shortening SN21 to only consist of the critical octapeptide stretch and a proline-to-alanine mutation led to the identification of P4A, which showed more potent macropinocytosis induction than SN21. P4A immediately converts to oxP4A (dominant form) and its dimers (P-P4A and A-P4A) under redox conditions in α -MEM, containing cysteine and cystine. Importantly, oxP4A induced a higher Dex70 uptake than P4A. Considering the loss of activity in the presence of serum, we are improving the applications of oxP4A in serum, and hopefully *in vivo*. Finally, we show that LK15-conjugated oxP4A delivers functional proteins into cells. More notably, LK15-oxP4A preferably delivered proteins tagged with negatively charged protein tags. The validity was exemplified through the efficient delivery of functional proteins fused with a supernegatively charged (–30)GFP at submicromolar concentrations (0.5 μ M). The strategies presented herein should aid in future developments of peptide-based biomacromolecule delivery strategies that enter via macropinocytosis.

Acknowledgments

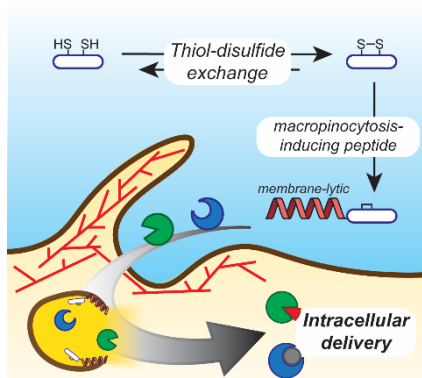
This work was supported by JSPS KAKENHI (Grant Numbers 18H04403 and 18H04017), and by JST CREST (Grant Number JPMJCR18H5). Y. H. is grateful for the JSPS Research Fellowship for Young Scientists. The authors thank Professors T. Itoh (Kobe University) and K. Nakayama (Kyoto University) for their gifts of lifeact-mCherry and pGEX-anti-mCherry Nanobody plasmids, respectively. The authors also thank Prof. Y. Hayashi (Tokyo University of Pharmacy and Life Science) for providing the NPyS-OMe reagent.

Keywords: Intracellular delivery • Macropinocytosis • Nanobodies • Peptides • Thiol-disulfide exchange

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Entry for the Table of Contents



Macropinocytosis is a ubiquitous cellular delivery route. This report identified a short macropinocytosis-inducing peptide P4A that undergoes thiol-disulfide exchange reaction, yielding a potent intramolecular oxidized analog (oxP4A). The conjugate of oxP4A and the membrane lytic peptide LK15 delivered functional proteins. Most notably, this peptide delivers proteins fused with a supernegatively charged protein tag at nanomolar concentrations.

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