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Activatable Cell Penetrating Peptide–Peptide Nucleic Acid Conjugate via Reduction of Azobenzene PEG Chains

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Supporting Information

ABSTRACT: The use of stimuli-responsive bioactive molecules is an attractive strategy to circumvent selectivity issues *in vivo*. Here, we report an activatable cell penetrating peptide (CPP) strategy ultimately aimed at delivering nucleic acid drugs to the colon mucosa using bacterial azoreductase as the local reconversion trigger. Through screening of a panel of CPPs, we identified a sequence (M918) capable of carrying a nucleic acid analogue payload. A modified M918 peptide conjugated to a peptide nucleic acid (PNA) was shown to silence luciferase in colon adenocarcinoma cells (HT-29-luc). Reversible functionalization of the conjugate's lysine residues via an azobenzene self-immolative linkage abolished transfection activity, and the free CPP-PNA was recovered after reduction of the azobenzene bond. This activatable CPP conjugate platform could find applications in the selective delivery of nucleic acid drugs to the colon mucosa, opening therapeutic avenues in colon diseases.

Cell penetrating peptides (CPPs) have the ability to carry a large diversity of cargos into cells, including macromolecules such as nucleic acids, which otherwise suffer from low efficacy due to their poor membrane permeation.[1] Despite their utility in drug delivery, the full clinical potential of CPPs has not yet been realized, in great part due to nonspecific tissue interaction of CPP *in vivo*, as well as toxicity issue associated with their cationic nature. Various approaches have been investigated to overcome these issues, such as addition of targeting ligands[2] or stimuli-triggered CPP activation.[3] Activatable CPPs have been developed by transiently masking the basic residues, known to be crucial for activity, or shielding the overall peptide with poly(ethylene glycol) (PEG). Depending on the delivery purpose and target site, various stimuli, such as pH,[3a] UV irradiation,[3c] and enzymes,[3b,d] have been applied to remove masking moieties from the system, resulting in the recovery of intact CPP activity. However, most of the systems tested in these applications were based on nanoparticulate carriers. Chemically well-defined molecular entities have the advantage of simpler and more reliable preparation, purification, and characterization as well as more intelligible structure–activity relationship.

In this work, we developed an activatable CPP conjugate platform for the delivery of nucleic acid drugs based on CPP inactivation through functionalization with a self-immolative azobenzene moiety (Figure 1). Azobenzene bonds are reduced in the colon by bacterial azoreductase produced by the microbiota.[4] Thus, azobenzene-containing molecules have been extensively used in colon-specific drug delivery systems such as prodrugs of 5-amino salicylic acid currently available on the market (sulfasalazine, balsalazine, etc.), hydrogels, and coating polymers.[5] Here, a CPP-nucleic acid conjugate is protected from nonspecific tissue interaction and absorption in the intestine by attaching PEG chains to the lysine residues of the CPP. The reductive cleavage of the azobenzene bond yields a self-immolative aminobenzyl carbamate that releases the native lysine and the 4-aminobenzyl alcohol, a byproduct with low cytotoxicity[6] (Figure 1, inset). Upon recovery of the intact structure, the CPP will promote the uptake of the nucleic acid in the colonic epithelial cells, leading to the inhibition of a protein of interest. The local delivery of nucleic acid drugs to the colon mucosa holds great promise for the treatment of inflammatory bowel diseases such as ulcerative colitis and Crohn's disease, as well as colon cancer.

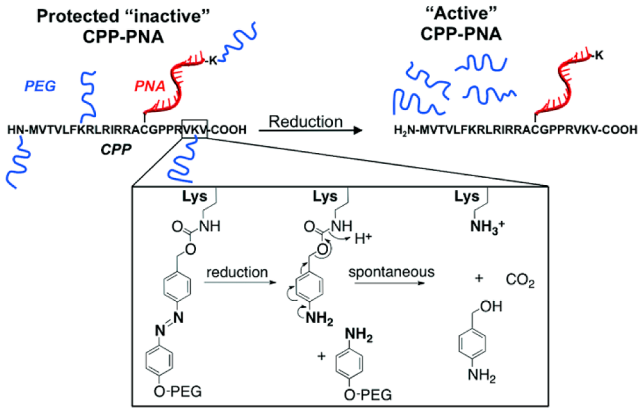

Figure 1. Schematic illustration of colon-specific delivery with activatable CPP-PNA (Inset: After the reductive cleavage of azobenzene bond, a self-immolative moiety releases the intact lysine residue on the CPP backbone).

We first aimed at selecting the appropriate nucleic acid and CPP. Unmodified nucleic acids are enzymatically labile and their oral administration usually requires encapsulation in a carrier. Among the numerous chemically modified nucleic acids known, peptide nucleic acids (PNAs) are especially suited for

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Table 1. Sequences and Nomenclature of Cell Penetrating Peptides

name	abbreviation	sequence[a]
Melittin	Mel	CIGAVLKVLTTGLPALISWIKRKRQQ
Transportan 10	TP10	AGYLLGKINLKALAALAKKILGGC
GKK peptide[b]	GKK	GKKALKLAAKLLKKC
R6-Pen$_{(W-L)}$	R6Pen	RRRRRRRQIKILFQNRRMKWKKGGC
ppTG-1	ppTG1	GLFKALLKLLKSLWKLLLKAGGC
MPG-Δ^{NLS}	MPG	GALFLGWLGAAGSTMGAPKSKRKVGGC
Pep-3	Pep3	KWFETWFTEWPKKRKGGC
EB-1	EB1	LIRLWSHLIHIWFQNRRLKWKKKGGC
KALA	KALA	WEAKLAKALAKALAKHLAKALAKALKACEA
M918	M918	MVTVLFRRLRIRRACGPPRVRV
M918$_{(C-S)}$[c]	M918S[19]	**C**GGMVTVLFRRLRIRRA**S**GPPRVRV-NH_2
M918$_{(R-K)}$[d]	M918K	MVTVLF**K**RLRIRRACGPPRV**K**V

[a]No modification at the peptide ends except for M918S that is amidated at C-terminus. [b]Amphipatic model peptide from systematic structure−activity relationship studies. [c]Cys residue was located at the N-terminus and the previous Cys was replaced with Ser (underlined and bold). [d]Two Arg in M918 were replaced with Lys (underlined and bold).

our application due to their resistance to enzymatic degradation.[7] Furthermore, the uncharged backbone of PNA prevents undesired interactions with the positively charged CPP, which would result in precipitation or inactivation of the CPP. For rapid and unbiased determination of post-transcriptional gene silencing, a PNA with a complementary sequence to luciferase mRNA was used in this study. A lysine residue was added to the end of the sequence as is commonly done to improve solubility (Materials section in Supporting Information (SI)). To verify the PNA sequence suitability, colon adenocarcinoma cells stably expressing luciferase (HT-29-luc) were transfected with PNA using streptolysin O (SLO) (SI Figure S1A,B).[8] The PNA specifically silenced luciferase expression compared to a mismatch PNA (mmPNA) control (SI Figure S1C). To select an efficient CPP, a library of CPP-PNA conjugates was synthesized using a selection of previously reported sequences. The delivery efficiencies of TP10,[9] GKK,[10] R6Pen,[11] M918,[12] Mel,[13] ppTG1,[14] MPG,[15] Pep3,[16] EB1,[17] and KALA[18] have been previously explored as part of PNA-conjugates or as electrostatic complexes with negatively charged nucleic acids. For conjugation to PNA, a cysteine residue in the peptide sequence was used, or a cysteine residue and diglycine linker was added to the N- or C-terminus of peptides (Table 1). All CPP-PNA conjugates were prepared via maleimide−thiol coupling reaction, purified by HPLC, and characterized by LC-MS (SI Table S1 and Figure S2).

In an initial screening, HT-29-luc cells were transfected with CPP-PNA conjugates at the maximum concentration that did not cause toxicity (SI Figure S3). Interestingly, only one CPP-PNA candidate, M918-PNA, showed a significant silencing of target luciferase protein even though some of them had demonstrated excellent delivery efficiencies in previous studies (Figure 2A). A possible reason may be cell line-dependent levels of CPP toxicity,[20] which limits the concentration that can be used (SI Figure S4). Another factor may be that most work on PNA conjugates involves pre-mRNA splicing correction system, which requires lower PNA concentrations than our model system, in which the PNA acts via a steric blocking mechanism.[11,12,19] Importantly, we studied the influence of endosome sequestration, which plagues several CPPs, by performing transfection in the presence of the lysosomotrophic agent chloroquine that enhances endosomal escape (SI Figure S5). Consistent with previous studies,[12] chloroquine treatment enhanced luciferase inhibition in most CPP-PNA transfections

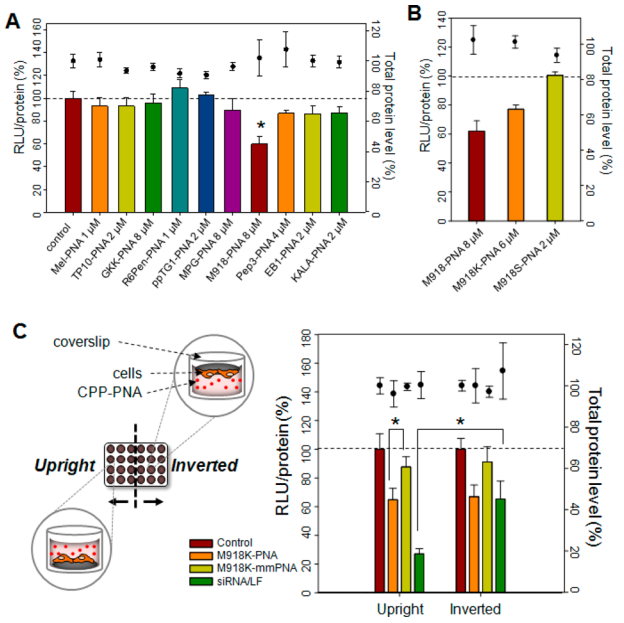

Figure 2. (A) Luciferase silencing efficiency (bar, left *y*-axis) and total protein level (filled dot, right *y*-axis) after transfection of various CPP-PNA conjugates. Values are represented as a mean ± SD (n = 3). *p < 0.001 vs control (medium treatment under the same conditions). (B) Luciferase silencing efficiency (bar, left *y*-axis) and total protein level (filled dot, right *y*-axis) after transfection with PNA conjugated with M918 variants. Values are represented as a mean ± SD (n = 3). (C) Illustration of upright and inverted transfection methods (left panel), and sequence specific luciferase silencing (bar, left *y*-axis) and total protein level (filled dot, right *y*-axis) with luciferase targeting PNA-M918K (M918K-PNA) or mismatch PNA-M918K (M918K-mmPNA) at 6 μM in upright and inverted transfection (right panel). Values are represented as a mean ± SD (n = 9, except for M918K-mmPNA n = 3). *p < 0.001.

at a concentration of 1 μM. This result explains the failure of most CPP-PNAs, which are entrapped in the endosome after intracellular uptake, and also suggests that the lack of activity of the conjugates is not due to the loss of PNA activity after covalent conjugation.

Since the most active CPP (M918) contains no lysine residues, we modified its sequence and replaced two cationic arginines by lysine residues (M918K, Table 1). To investigate the influence of PNA coupling site, we also shifted the cysteine

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residue used for conjugation from the middle of the sequence to its N-terminus (M918S, Table 1). The luciferase silencing efficiency of the M918K-PNA conjugate was comparable to that of M918-PNA, suggesting that the lysine substitution was tolerated. However, M918S-PNA showed unexpectedly high toxicity (Figure 2B and SI Figure S3 and S6). Interestingly, the free M918S peptide also showed higher toxicity than both M918 and M918K (SI Figure S4B). This phenomenon has also been observed by El-Andaloussi *et al.*, who demonstrated that TP10 labeled with fluorescein at the N-terminus showed higher toxicity than when labeled in the middle of peptide.[21] On the basis of these results, we selected M918K-PNA for further study.

By performing transfection experiments with upside down cells, Cho *et al.* revealed that the *in vitro* transfection of many nanoparticles is artificially promoted by sedimentation.[22] The luciferase-silencing efficacy of M918K conjugates was evaluated in a conventional upright and in an inverted transfection setup (Figure 2C). The transfection efficiency of M918K-PNA was not altered by changing the position of cells from the bottom (upright transfection) to the top of transfection medium (inverted transfection). However, the transfection efficiency of siRNA-lipofectamine (LF) complex decreased dramatically in inverted transfection due to decrease of cellular uptake.[22b] This result highlights a potential advantage for *in vivo* applications of a well-defined single-molecule entity as opposed to nanoparticulate delivery systems whose transfection efficiency are probably overestimated in conventional *in vitro* assays. Furthermore, the sequence-specificity of the silencing was demonstrated using a conjugate with a mismatch PNA sequence (M918K-mmPNA), which showed no significant luciferase silencing.

We then sought to transiently inactivate the CPP-PNA by conjugating the lysine residues that are important for their transduction activity to PEG. Furthermore, the addition of PEG chains results in a larger molecule, thus reducing nonspecific interactions with the intestinal mucosa and absorption during transit through the small intestine. A small PEG chain length (M_n 750) in line with previous work[23] was used to avoid potential steric inhibition of azoreductase. Thus, a PEGylated azobenzene incorporating a latent self-immolative aminobenzyl moiety was synthesized by a Mills coupling reaction (SI Scheme S1).[24] The CPP-PNA PEGylation was performed with excess of the corresponding activated carbonate **1** (Figure 3). SDS-PAGE analysis of the PEGylation reaction revealed a new band with increased molecular weight (Figure 4A). No band at the position of free M918K-PNA was detected, indicating that most of M918K-PNA was successfully PEGylated. Consistent with this result, MALDI-MS analysis showed no peak corresponding to the molecular weight of free M918K-PNA (Figure 4B, SI Figure S7A,B). In contrast to SDS-PAGE, MALDI could resolve the individual PEGylated products, with broad peaks separated by the molecular weight of the PEG-azobenzene moiety. M918K-PNA contains 3 lysine residues and the N-terminal methionine; consequently, 4 peaks could be observed, with conjugates containing 3−4 PEG chains as major products.

Since active azoreductase enzymes are not commercially available, the reconversion of the PEGylated conjugate to the free M918K-PNA was studied with the surrogate reducing agent sodium dithionite.[25] After incubation with sodium dithionite, SDS-PAGE analysis showed disappearance of the PEGylated conjugate band and the reappearance of the free

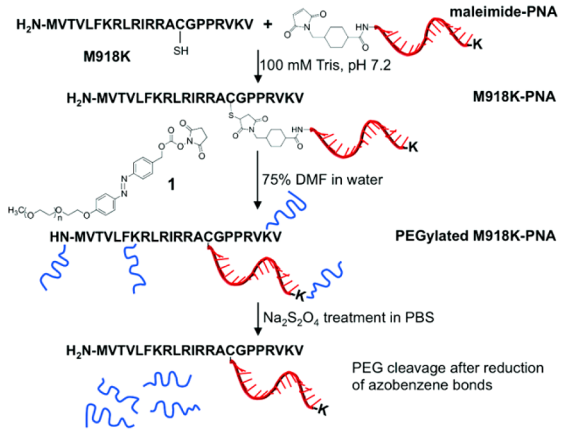

Figure 3. Synthetic scheme of PEG-azobenzene conjugation to the N-terminal or Lys residues on CPP-PNA backbone and the cleavage of PEG by reduction.

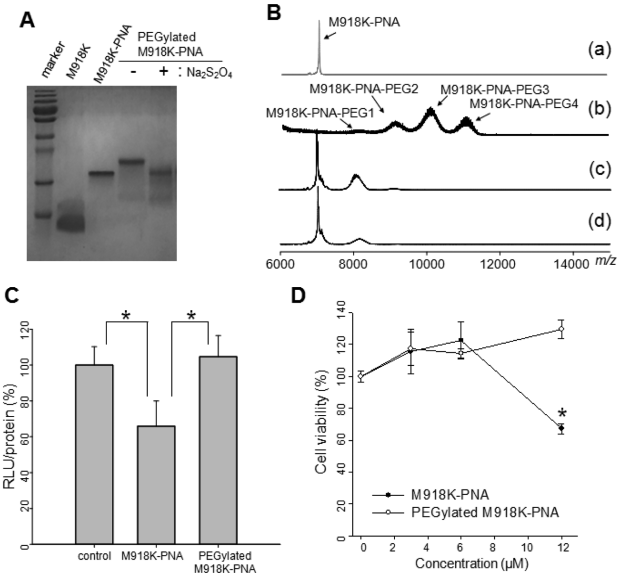

Figure 4. (A) SDS-PAGE of M918K, M918K-PNA and PEGylated M918K-PNA with or without $Na_2S_2O_4$ treatment. (B) MALDI-MS of (a) M918K-PNA, (b) PEGylated M918K-PNA, (c) PEGylated M918K-PNA treated with 72 mM of $Na_2S_2O_4$ for 15 min, and (d) PEGylated M918K-PNA treated with 574 mM of $Na_2S_2O_4$ for 2 h. (C) Luciferase silencing assay with 6 μM of M918K-PNA and PEGylated M918K-PNA. (D) Cell viability test with M918K-PNA and PEGylated M918K-PNA at different concentrations. Values are represented as a mean ± SD ($n = 3$) *$p < 0.05$ vs control (medium treatment under the same conditions).

M918K-PNA band (Figure 4A). Furthermore, MALDI-MS was performed with PEGylated M918K-PNA treated with sodium dithionite at different concentrations (Figure 4B, SI Figure S7C,D). The peak corresponding to molecular weight of intact M918K-PNA reappeared, while the peaks corresponding to M918K-PNA conjugated with more than 2 PEG chains disappeared. M918K-PNA containing one PEG chain was still observed after 72 mM sodium dithionite treatment but marginally detected after 8-fold higher amount of sodium dithionite treatment. The small amount of mono-PEGylated CPP-PNA remaining after the reaction is most probably due to the inactivation of the dithionite by oxidation.[25d] It should be

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noted that the lability of the azobenzene moiety can be tailored by tuning the electronic properties of the aromatic rings.[25a,b]

Having demonstrated the reversibility of the PEGylation, we then compared the transfection efficiency of PEGylated M918K-PNA to that of free M918K-PNA in order to confirm that neutralization of the M918K lysines produced an inactive conjugate (Figure 4C). As anticipated, PEGylation caused a total loss of activity in HT-29-luc cells. Additionally, the effect of both conjugates on cell viability was compared because CPPs are cytotoxic at high concentrations. Whereas PEGylated M918K-PNA treatments up to 12 μM did not change the cell viability, M918K-PNA elicited toxicity at this high concentration, thus reaffirming the passivation of the CPP moiety (Figure 4D).

In summary, a novel activatable CPP−nucleic acid drug conjugate strategy was developed. A CPP sequence capable of delivering an active PNA payload was identified through screening of a panel of conjugates. Surprisingly, a majority of known CPP moieties were shown to be inactive, due in part to endosomal sequestration after uptake. However, the M918K-PNA conjugate showed sequence-specific silencing of luciferase *in vitro*, which was not artificially promoted by sedimentation. Furthermore, reversible PEGylation of the conjugate's lysine residues completely abolished transfection activity. Reconversion of the PEGylated conjugate to the free M918K-PNA could be effected by reduction of the diazobenzene moiety. This activatable CPP−nucleic acid drug delivery platform could constitute an interesting vehicle not only to modulate known disease-causing gene expression but also to explore new therapeutic targets in colon diseases.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Experimental section and supporting results. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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