UNIVERSITY OF COPENHAGEN
DEPARTMENT OF CHEMISTRY

Peptide-Oligonucleotide Conjugates
Effect on Endosomal Escape?

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Abstract

RNA- and DNA-based oligonucleotide (ON) therapeutics are transforming disease treatment due to their high specificity and clinical efficacy. However, their therapeutic potential remains limited by inefficient intracellular delivery.[1] Following cellular uptake, most of the ONs are entrapped in endosomal compartments and undergo lysosomal degradation. Only a small fraction (estimated < 4%) of internalized RNA successfully escapes into the cytosol to reach their target. [2] Although several mechanisms of endosomal escape have been proposed, their molecular basis remains incompletely understood. Peptide-based ONs strategies have been explored to enhance endosomal escape, yet efficiency-toxicity trade-offs persist.[2]

This project aims to systematically investigate the mechanisms governing endosomal escape through the design of multifunctional peptide-oligonucleotide conjugates. This will be supported by real-time single particle molecular microscopy of labelled oligonucleotide-peptide conjugates in living cells, isolated endosomes and membrane models. This approach reveals how individual conjugates interact with membranes, move through cells and escape from endosomes, providing mechanistic insight into the factors governing efficient intracellular delivery and functional release

1. Intracellular Uptake of Oligonucleotides

Following cellular uptake by endocytosis, ONs are transported from early endosomes (pH 6.0-6.5) to late endosomes (pH 5.0-6.0) and lysosomes (pH 4.5-5.0). The increasing acidification and changes in membrane composition promote cargo sorting and degradation of the ONs.[2]

Peptide conjugation may enhance uptake and interact with endosomal membranes to promote endosomal escape into the cytosol. These include cell-penetrating peptides (CPPs), hydrophobic endosomal escape domain peptides (EEDs), and pH-responsive peptides. However, identifying peptides that selectively disrupt endosomal membranes without causing toxicity remains a challenge.

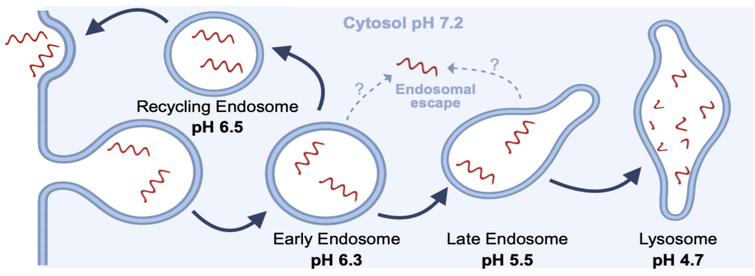


Figure 1 The intracellular pathway and trafficking of ONs and the great endosomal escape

2. Fluorophore Labelling of Antisense Oligonucleotides

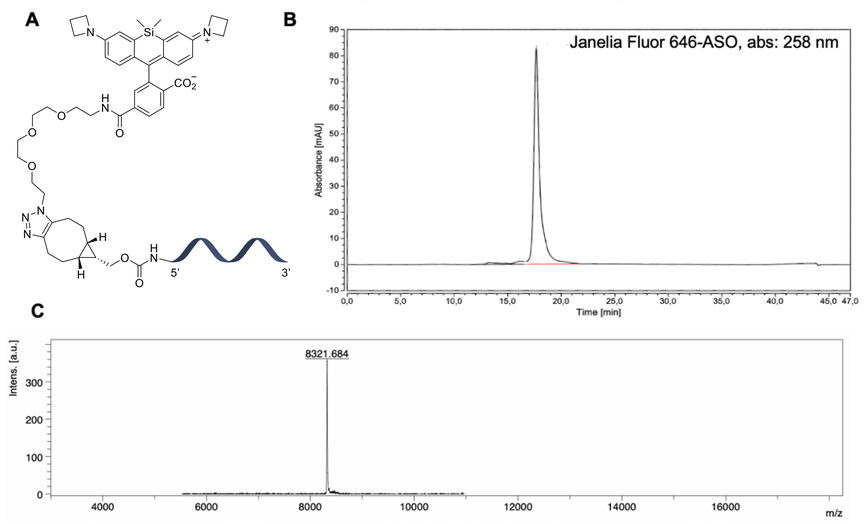


Figure 2 **Janelia Fluor 646 – MALAT1_ASO (A)** Structure of Janelia Fluor 646 – MALAT1_ASO **(B)** HPLC chromatogram (258 nm) of Janelia Fluor 646-MALAT1_ASO, obtained on Vanquish UHPLC equipped with a C18 column (250 x 10 mm from Phenomenex) at 60 °C. **(C)** MALDI-TOF MS obtained on Bruker Mircroflex calcd. [M+H⁺] 8318.8; m/z found 8321.684.

3. Fluorophore Labelled Antisense Oligonucleotides are Heterogeneously Distributed and Sequestered Within the Endolysosomal Lumen [6]

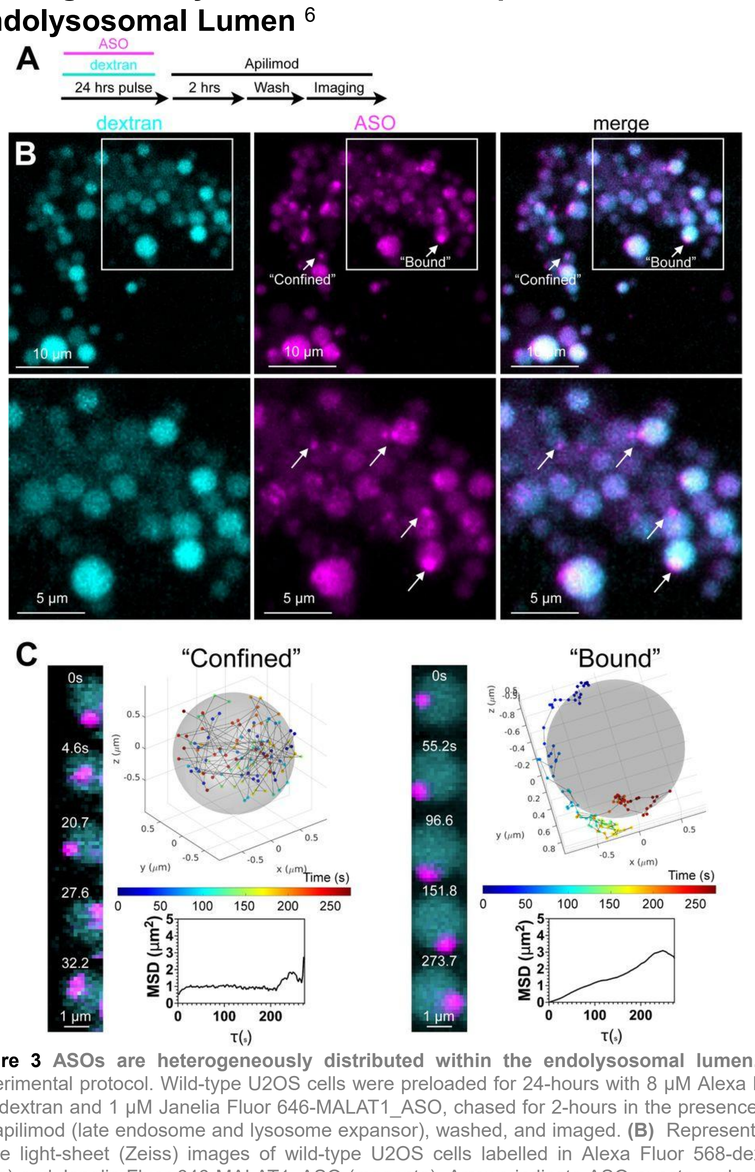
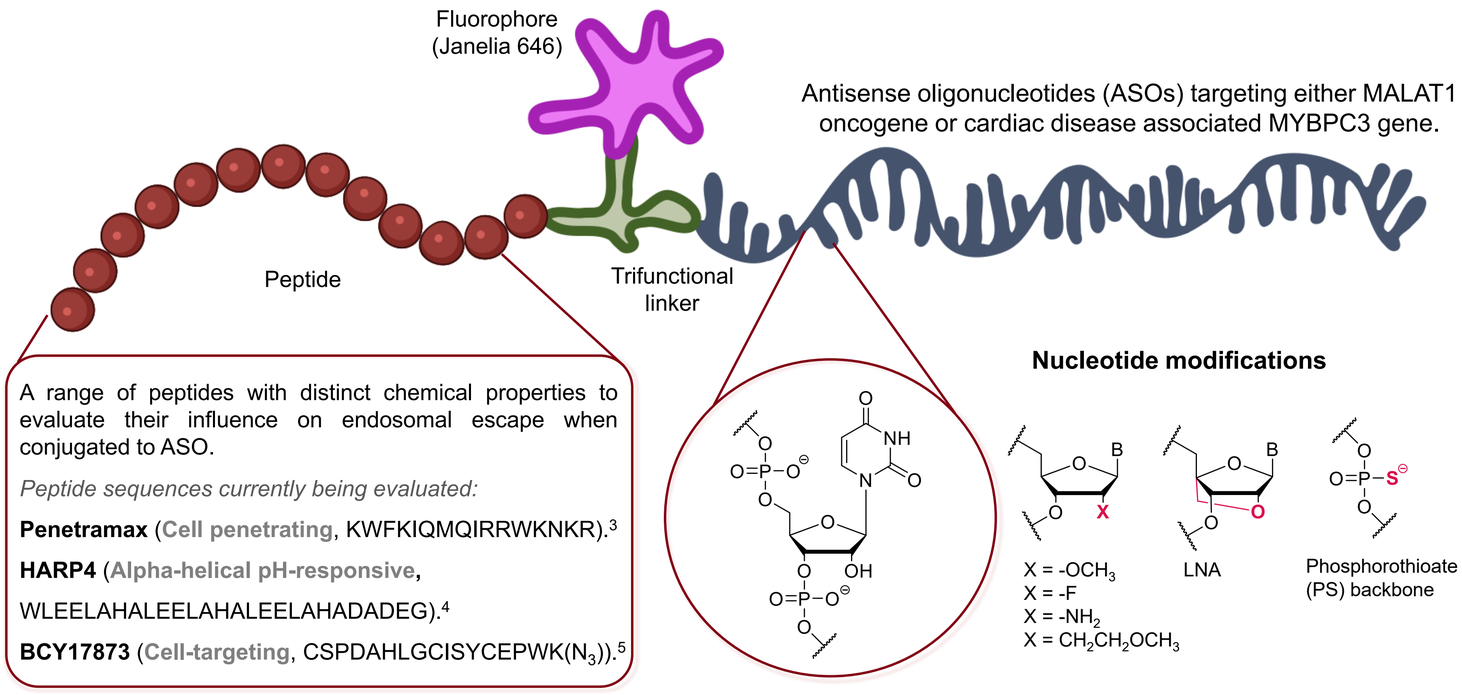


Figure 3 **ASOs are heterogeneously distributed within the endolysosomal lumen. (A)** Experimental protocol. Wild-type U2OS cells were preloaded for 24-hours with 8 µM Alexa Fluor 568-dextran and 1 µM Janelia Fluor 646-MALAT1_ASO, chased for 2-hours in the presence of 5 µM apilimod (late endosome and lysosome expansor), washed, and imaged. **(B)** Representative lattice light-sheet (Zeiss) images of wild-type U2OS cells labelled in Alexa Fluor 568-dextran (cyan) and Janelia Fluor 646-MALAT1_ASO (magenta). Arrows indicate ASO puncta undergoing confined and bound motion. **(C)** Representative real time three-dimensional trajectories of individual ASO particles exhibiting confined or bound motion within endolysosomal compartments. Mean squared displacement (MSD) plots are shown for each trajectory. Data are representative from one of three independent experiments.

4. Design of Oligonucleotide Conjugates for Evaluation of Endosomal Escape



A range of peptides with distinct chemical properties to evaluate their influence on endosomal escape when conjugated to ASO.

Peptide sequences currently being evaluated:

Penetramax (**Cell penetrating**, KWFKIQMQIRRWKNKR).[3]

HARP4 (**Alpha-helical pH-responsive,**

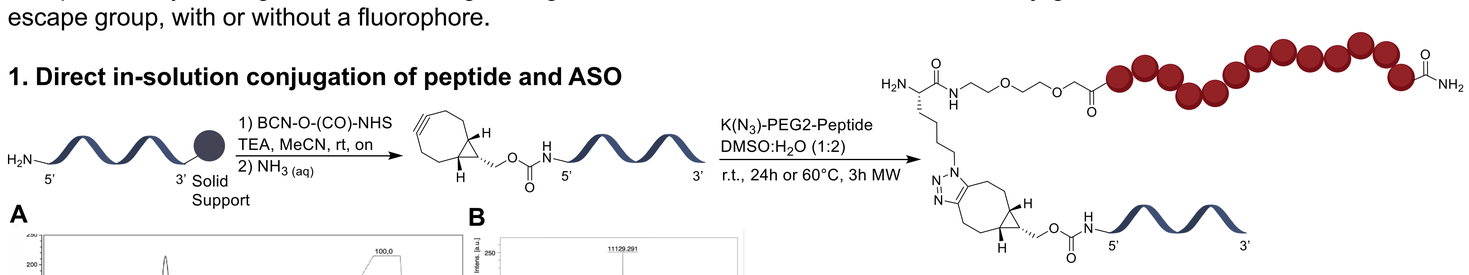
WLEELAHALEELAHALEELAHADADEG).[4]

BCY17873 (**Cell-targeting**, CSPDAHLGCISYCEPWK(N_3)).[5]

5. Peptide-Oligonucleotide Conjugation: Our Synthetic Strategies

Complementary strategies for introducing orthogonal chemical handles to enable the conjugation of an ASO to an endosomal escape group, with or without a fluorophore.

1. Direct in-solution conjugation of peptide and ASO



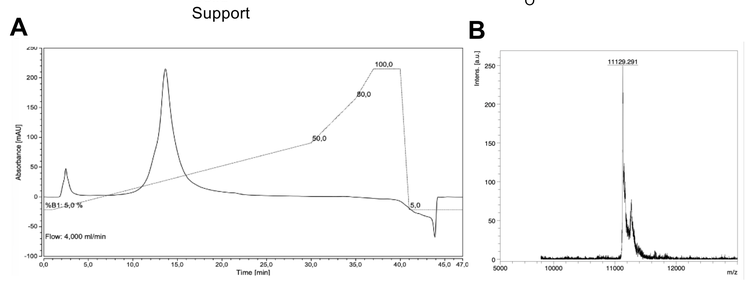
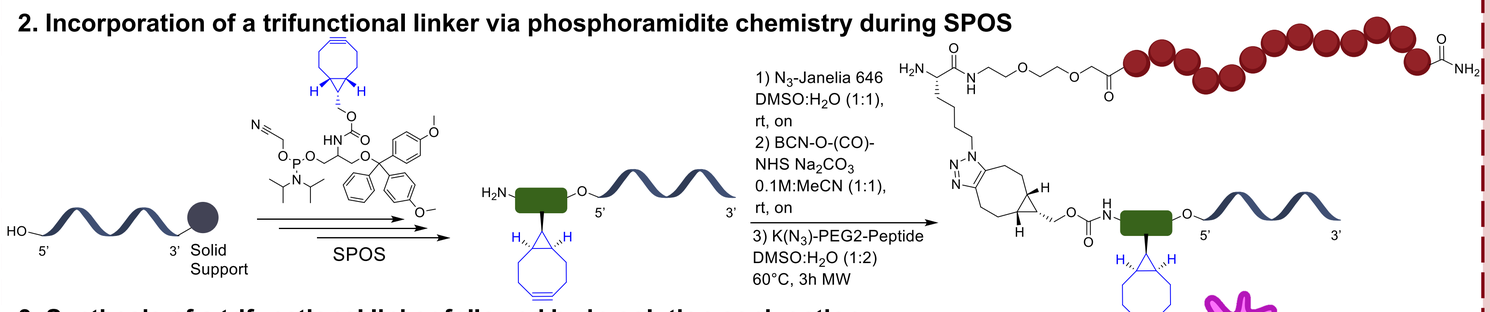
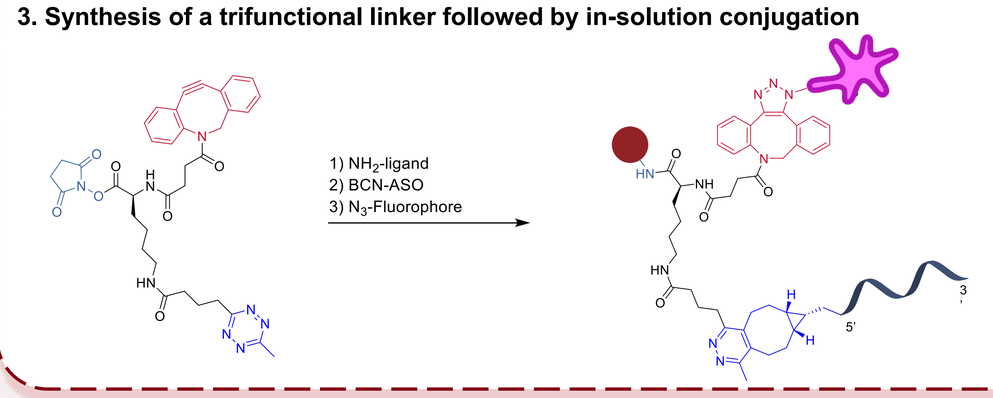


Figure 4 **(A)** HPLC chromatogram (258 nm) of Penetramax-ASO, obtained on Vanquish UHPLC equipped with a C18 column (250 x 10 mm from Phenomenex) at 60 °C. **(B)** MALDI-TOF mass spectrometry of Penetramax-ASO, obtained on Bruker Mircroflex calcd. [M+H⁺] 11130.01; m/z found 11129.291.

2. Incorporation of a trifunctional linker via phosphoramidite chemistry during SPOS



3. Synthesis of a trifunctional linker followed by in-solution conjugation



6. Concluding Remarks

- We have established different synthetic routes for peptide-ASO conjugates, both via direct in-solution conjugation and through a trifunctional linker enabling an additional conjugation group.
- Successful conjugation of three distinct peptides to ASO; CPP (Penetramax), pH-responsive membrane targeting (HARP4), and cell-targeting (BCY17873), confirmed by HPLC and MALDI-TOF MS and are being evaluated for their effects on endosomal escape.
- ASOs were successfully detected within the endosomes
- Live-cell imaging reveals heterogeneous ASO distribution within the endolysosomal lumen, with particles exhibiting both confined and bound motion.
- Future work will focus on evaluating endosomal escape efficiency across peptide-ASO conjugates

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References: **(1)** S. Azari et al., *Int. J. Pharm.* **2026**, 687, 126393, **(2)** S. F. Dowdy, *RNA.* **2023**, 29, 396–401, **(3)** R. G. Diedrichsen et al., *J. Control. Release.* **2025**, 380, 1031–1042, **(4)** H. Sakurai et al., *ACS Appl. Bio Mater.* **2021**, 4, 2442–2452. **(5)** M. E. Østergaard et al., *Nucleic Acids Res.* **2025**, 5 **(6)** E. Sitarska et al., bioRxiv **2026**, 2026.05.11.724403. **(7)** C. Lou, et al., *Chem. Eur. J.* **2020**, 26, 5676–5684.

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