P-393

Application of Antibody Site-Specific Modification Method tCAP to Brain-Penetrating Antibody-Oligonucleotide Conjugate

○Yuji Ito[1], Abdur Rafique[1], Hiroko Kawakami[1,2], Taku Yoshiya[2,3]

[1] Graduate School of Science and Engineering, Kagoshima University, Kagoshima, Japan
[2] Peptide Institute, Inc., Osaka, Japan.
[3] Institute for Protein Research, The University of Osaka, Osaka, Japan
Email: yito@sci.kagoshima-u.ac.jp, https://doi.org/10.17952/38EPS.2026.P393

Abstract

We developed a site-specific IgG modification method, tCAP, which employs an Fc-binding affinity peptide to induce proximity-driven introduction of an azide group at Lys248 on the Fc region.[1,2] Site-specific modification is achieved simply by mixing native antibodies with the tCAP reagent. Here, we report the preparation of antibody–oligonucleotide conjugates (AOCs) using this method and their brain delivery in mice.

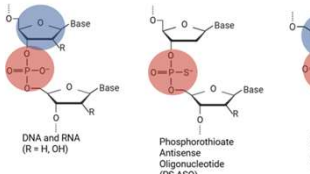
An anti-TfR VHH-Fc antibody with BBB-penetrating capability and reaching to neuronal cells[3] was modified with an azide group using tCAP, followed by click conjugation with IR-800-labeled DBCO-oligonucleotides (PS or PMO types) added at threefold molar excess. LC–MS analysis confirmed a bivalent AOC (DAR = 2.0) with approximately 95% purity.

After intravenous administration in mice, brain fluorescence imaging and microscopy at 24, 48, and 96 h demonstrated that PMO-modified AOCs achieved ~70% of the brain uptake of the unconjugated antibody and reached neuronal cells in the brain parenchyma. In contrast, PS-modified AOCs showed <3% uptake, and monovalent PS-AOCs did not improve brain penetration.

These findings indicate that the physicochemical properties of oligonucleotides, particularly charge characteristics, critically affect AOC transport from blood to brain. Further optimization, including linker design, will be required to enhance BBB penetration of PS-modified AOCs.

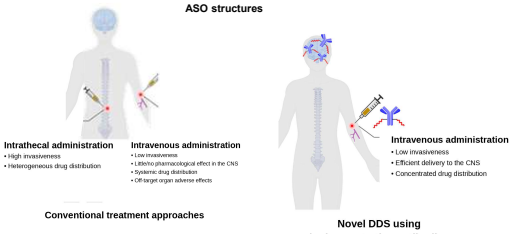
Background

• Antisense Oligonucleotide (ASO)

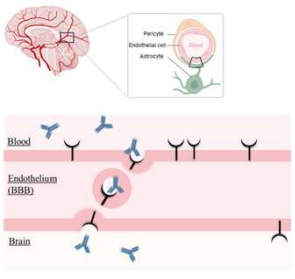


Modification	Backbone / charge	Key advantages	Main limitations
PS ASO	• Phosphorothioate backbone • Negatively charged	• High nuclease resistance • High membrane permeability	• Potential cytotoxicity
PMO	• Morpholine ring + phosphorodiamidate linkage • Charge-neutral	• High nuclease resistance • Low cytotoxicity	• Low membrane permeability

ASO structures



• Blood–Brain Barrier (BBB)



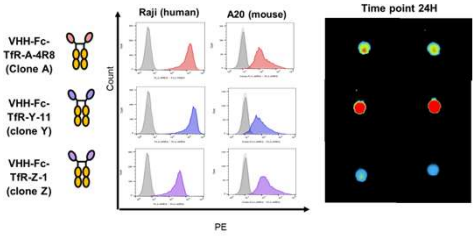
The blood–brain barrier (BBB) separates the blood from the brain and restricts the entry of substances into the brain.

Antibody therapeutics and many other drugs cannot efficiently reach the brain.

Brain delivery using anti-TfR antibodies targeting the transferrin receptor (TfR) expressed at the BBB.

TfR-mediated antibody transport across the BBB

• Isolation of anti-TfR VHH antibodies



TfR-mediated antibody transport across the BBB

• Fc site-specific modification: tCAP

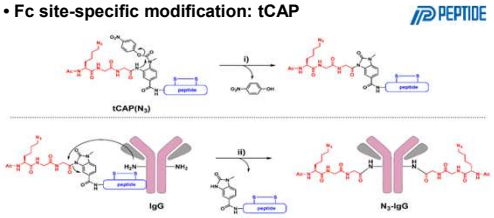


Fig. tCAP method

The tCAP method is an Fc site-specific antibody modification technology developed in our laboratory using a human IgG-affinity peptide. In the tCAP reagent, an azide group is linked to an Fc-specific antibody-affinity peptide through an activated acyl-donor moiety, enabling introduction of two azide groups into the Fc region of IgG. The introduced azide groups can subsequently be coupled to diverse molecules, including fluorescent probes and oligonucleotide therapeutics, by click chemistry with DBCO groups.

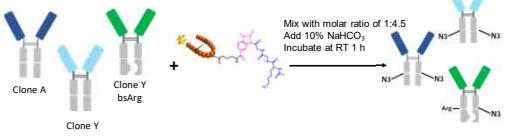
Methods

• Preparation of antibody–ASO conjugates (AOCs)

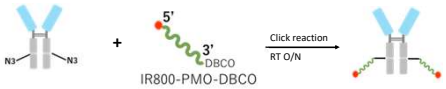
1. DBCO and IR800 labeling of PMO and ASO



2. VHH-TfR (Clone A, Y, bsArg) hFcm + tCAP reagent



3. VHH-TfR(Clone A, Clone Y) tCAP(N3) + DBCO-PMO-IR800



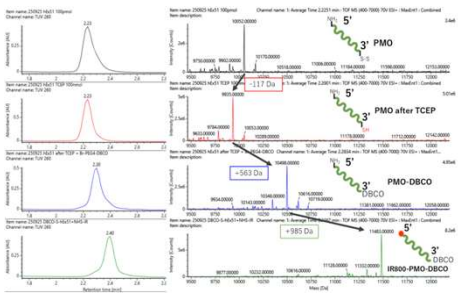
4. VHH-TfR(Clone Y bsArg) tCAP(N3) + DBCO-PS ASO-IR800



Results & Discussion

• Preparation of antibody–ASO conjugates (AOCs)

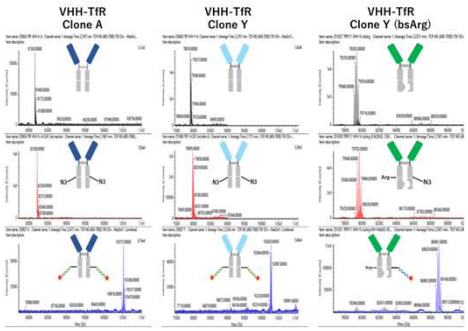
1. DBCO and IR800 labeling of PMO



Confirmation of DBCO and IR800 labeling of PMO by Waters BioAccord LC-MS

PMO was reacted with TCEP·HCl at a 1:10 molar ratio and purified using a Sephadex G-25 column. Br-PEG4-DBCO was then reacted at pH 8.9. After confirming the reaction, NHS-IR800 was added, and the product was purified using a Sephadex G-25 column.

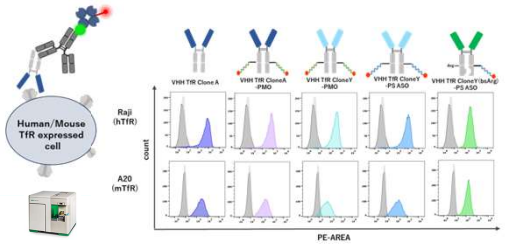
2. Preparation and characterization of AOCs using the tCAP method



Various ASO conjugates prepared by the tCAP method

Anti-TfR antibodies bearing azide groups introduced by the tCAP method were mixed at a 1:3.5 molar ratio with DBCO/IR800-labeled ASO and conjugated by click chemistry.
Unreacted ASO was removed using a Sephadex G-75 column. TOF-MS analysis confirmed formation of the desired AOCs with either bivalent or monovalent ASO conjugation.

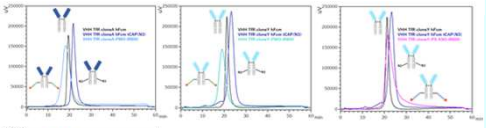
• TfR-binding activity by flow cytometry



Binding activity of AOCs assessed by flow cytometry

The binding activity of the prepared AOCs was evaluated using human and mouse TfR-expressing cells. Reduced binding was observed for VHH TfR clone Y-PMO and VHH TfR clone Y-PS ASO.

• rFcRn binding activity

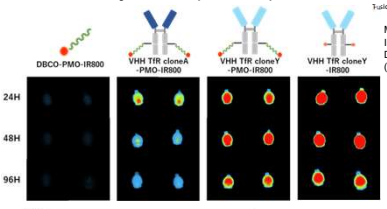


Evaluation of rFcRn binding using an rFcRn affinity column

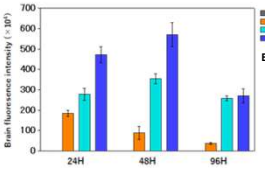
To evaluate FcRn binding of the prepared AOCs, we used a column (TOSOH) immobilized with recombinant FcRn.
For all antibodies, tCAP modification resulted in stronger binding than the unmodified antibody. All AOCs showed binding comparable to the corresponding unmodified antibodies, indicating that FcRn binding activity was retained after AOC formation.



• Brain delivery of AOC (Ab–PMO)



Mouse: male ICR
Injection: tail vein
Dose: 50 nmol/kg (PMO: 100 nmol/kg)

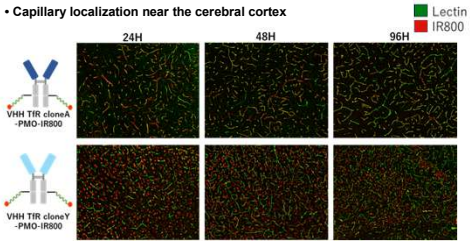


Brain delivery evaluation of AOC (PMO)

AOCs were prepared by conjugating PMO to VHH TfR clone A or clone Y, and brain delivery was evaluated.
Clone Y AOC showed higher brain fluorescence than clone A AOC. At 24 and 48 h, the fluorescence intensity of clone Y AOC was approximately 60% of that of clone Y alone.

• Brain localization of AOC (Ab–PMO)

• Capillary localization near the cerebral cortex



Localization of AOC (Ab–PMO) evaluated using Lectin488

At each time point for VHH TfR clone A-PMO and clone Y-PMO, brain capillaries were stained with Lectin488 and compared with IR800 fluorescence. In the clone A-PMO group, IR800 fluorescence was localized mainly to capillaries.
In contrast, clone Y-PMO showed a different distribution, with IR800 fluorescence located away from capillaries.

• Neuronal localization near the cerebral cortex

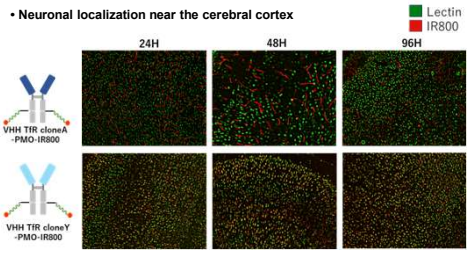
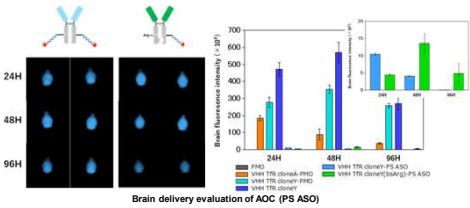


Fig. Localization of AOC (Ab–PMO) evaluated by NeuN staining

At each time point, neurons were stained with an anti-NeuN antibody and a secondary antibody and compared with IR800 fluorescence. For VHH TfR clone Y-PMO, IR800 fluorescence overlapped with NeuN-positive signals, indicating that PMO could cross the BBB and reach neurons.

• Brain delivery of AOC (Ab–PS ASO)



Brain delivery evaluation of AOC (PS ASO)

Mouse: male ICR (7–8 weeks)
Injection: tail vein
Dose: 50 nmol/kg
(bsArg AOC: 100 nmol/kg to normalize IR800 valency)
Time points: 24, 48, 96 h

Brain delivery of bivalently and monovalently conjugated AOC (PS ASO) was evaluated. Neither showed appreciable brain delivery compared with the PMO-conjugated AOCs.

Summary

Two anti-TfR VHH antibodies (clone A and clone Y) were conjugated with PMO or PS ASO to generate AOCs, and their brain delivery and localization were evaluated. The VHH TfR clone Y-PMO conjugate showed high brain delivery and was detected in neurons. In contrast, PS ASO-conjugated VHH TfR clone Y showed little brain delivery compared with the PMO conjugate; however, monovalent PS ASO conjugation tended to provide greater brain delivery than bivalent conjugation.
These results suggest that brain-penetrating antibodies can serve as carriers for CNS delivery of oligonucleotide therapeutics.

References

1. H. Kawakami, T. Yoshiya, Y. Ito et al., Chem. Eur. J., 2026, 32, e03144. DOI: 10.1002/chem.202503144.
2. K. Tsuji, Y. Ito, T. Yoshiya, H. Tamamura et al., ChemMedChem, 2026, 21, e202500820. DOI: 10.1002/cmdc.202500820.
3. F. Jatnika, A. Nagashima, W. Miyahara, A. Rafique, Y. Ito, *under submission.*