

Delivery of Antisense Oligonucleotide through Chemical Conjugation with Designed Short Non-Cationic Cell Penetrating Peptide using Click Chemistry for Duchenne Muscular Dystrophy

3.1 Abstract

Duchenne muscular dystrophy (DMD) is a fatal X-linked neuromuscular disease caused by frame shift mutations in the gene encoding dystrophin. 2'-O-methyl phosphorothioate (2OMePS) serves as an antisense RNA platform clinically used in DMD patients to facilitate exon skipping and production of an internally truncated, yet functional dystrophin protein. Peptide-conjugated antisense oligonucleotides (ASOs) offer a promising next-generation platform, where a cell-penetrating peptide (CPP) is linked to the 2OMePS backbone to enhance cellular uptake. Herein, we designed and synthesized new non cationic short CPP sequence that can be efficiently conjugated with the negatively charged 2OMePS ASO backbone using click chemistry. Several tailored conjugation strategies were employed, resulting in the efficient and cost-effective coupling of synthesized peptides with ASOs. Conjugation of the lead peptide ETWWK to 2OMePS ASO resulted in significant cellular internalization with precise nuclear localization of the ASO cargo. Studies suggest almost 2 fold upregulation of dystrophin mRNA expression for ETWWK-ASO construct in comparison to untagged ASO. Our findings suggest that the identified peptide holds promise for facilitating ASO delivery at sites of splicing, and can eventually be a therapeutic avenue for future ASO based DMD treatments.

3.2 Introduction

Duchenne muscular dystrophy (DMD) is a rare, progressive, and fatal X-linked muscle wasting disorder arising from mutation in the DMD gene, which encodes dystrophin detailed in introduction chapter under segment 1.1 and 1.2.¹⁻⁶

This chapter focuses on the development of novel, short, non-cationic cell-penetrating peptides (CPPs) designed to efficiently deliver macromolecular antisense oligonucleotides (ASOs) directly to the nuclear compartment, targeting their intended site of action. Unlike conventional CPPs rich in positively charged residues, our approach emphasizes the design of peptides with reduced cationic content to minimize toxicity and improve delivery efficiency. The therapeutic relevance of ASOs in Duchenne muscular dystrophy (DMD) is increasingly recognized, particularly in light of insights gained from the milder Becker muscular dystrophy (BMD). While DMD arises from out-of-frame or nonsense mutations in

the DMD gene, disrupting the mRNA reading frame and leading to diminished or absent dystrophin production.³⁻⁵ In contrast, a milder phenotype of DMD is Becker muscular dystrophy (BMD), is a muscle wasting disorder caused due to mutations in the same gene, yet maintaining the open reading frame and allows the production of an internally truncated, but partially functional dystrophin. Thus, restoration of the reading frame in DMD patients could lead to production of a shorter, partially functional dystrophin protein similar to that observed in BMD patients.^{5, 7} Exon skipping strategies employing antisense oligonucleotides (ASOs) offer a promising approach to restore the DMD mRNA reading frame this results in the translation of an internally truncated, yet functional, dystrophin protein.^{8, 9}

These ASOs target specific exons during pre-mRNA splicing, inducing skipping of the target mutated exons. For DMD, the advanced exon-skipping ASOs are the negatively charged 2'-O-methyl phosphorothioate (2OMePS) and the neutral phosphorodiamidate morpholino oligomers (PMOs).¹⁰ In that regard, PMO based ASO therapeutic, eteplirsen (Exondys 51) has got approval from the FDA for treating DMD patients with mutation in exon 51.¹¹ As, PMO and 2OMePS were more comparable for human exons, also, the findings suggest that PMOs might exhibit lower sequence specificity compared to 2OMePS.¹² This observation guided us for the choice of 2OMePS based ASO for targeting exon 51 of the DMD population. Herein, previously reported 2OMePS based ASO sequence targeting exon 51 was outsourced containing sequence **UCA AGG AAG AUG GCA UUU CU** attached with amino C-6 CED phosphoramidite linker at 5' end. LCMS calculated for ASO sequence **C₂₁₇H₂₈₉N₇₇O₁₂₁P₂₀S₂₀**, 7172.79(*calc.*), observed 7173. The empirical formula and mass of the synthesized ASO sequence clearly indicates the presence of PS and OMe modifications in every position of the ASO. The widespread application of ASOs is limited by challenges associated with their delivery to target tissues, primarily due to poor bioavailability.^{13,14} Current ASO-based therapies requires weekly dosing to attain and sustain clinical improvement in DMD, thus focusing the need for reduced dosing frequency is a desirable aspect.¹⁵ To tackle this challenge, Cell-penetrating peptides CPPs have emerged as one of the most promising delivery strategies among the various methods investigated where a CPPs is linked to the ASO,¹⁶⁻¹⁷ aiming to enhance cellular uptake, exon skipping, and dystrophin levels.¹⁸⁻²¹

Most of the designed CPPs are rich in arginine and lysine residues, which raises concerns regarding potential toxicity and limits their bio-distribution due to nonspecific binding.²²⁻²⁴ Secondly, previously studied CPPs have shown promise in improving the delivery of PMOs, they may not be suitable for delivering 2OMePS due to the tendency of highly positively charged CPPs to form strong aggregates when combined with the anionic 2OMePS ASO backbone.²⁵ Thus, the importance of non-arginine-containing CPPs with excellent cell-penetrating ability becomes apparent. Therefore, our CPP is designed with the intention of circumventing the abundance of arginine residues commonly employed in CPPs to enhance cellular internalization potential. In the current study, we designed and synthesized CPPs by exploring the role of dual tryptophan moiety in cell penetration ability while avoiding excess presence of highly cationic amino acids.²⁶ These peptides were later conjugated to negatively charged 2OMePS ASO backbone. This allowed the identification of a lead short 5-mer non-cationic peptide stretch ETWWK, which, upon conjugation to a 2OMePS ASO, demonstrated significant cellular uptake, where ETWWK conjugated ASO shows substantial cellular uptake in comparison to untagged ASO.

Next, in our study we developed a customized conjugation strategy to enable a straightforward and efficient coupling of CPPs with a 2OMePS ASO backbone using Cu(I)-catalyzed click chemistry. To date, limited work has been conducted on employing Cu(I)-catalyzed click chemistry for conjugating CPPs with PS-ONs. Choice of click chemistry over other bio conjugation techniques such as, thiol-maleimide,²⁷ native ligation,²⁸⁻³⁰ were employed in this study as click chemistry based bio conjugation has garnered much attention because of its high chemoselectivity, bio-orthogonal nature, and the superior metabolic stability.³¹⁻³² Additionally, the triazole linkage formed via click chemistry offers superior metabolic stability compared to alternatives like thiazoles formed by NAT bioconjugation, oxazoles, and maleimides.³² Firstly, we avoided the use of any expensive linkers to functionalize our ASO and CPPs with alkyne and azide motifs respectively. Herein, CPPs N-terminal amine was readily transformed into N-terminal azide unit via simple lab synthesized azo transfer reagent,³³ which were directly utilized in conjugation with alkyne unit of ASO to form triazole linkage, and thus enabling chemical conjugation of the CPP with ASO. Next, the synthesized alkyne linker attached to the ASO fragment was short and active, facilitating efficient conjugation. In this process, we use CuBr × Me₂S as a source of Cu(I), instead of copper iodide, which intensely reduces the scope of cleavage and desulfurization of PS linkages in presence of metal ions. This evades one of the drawbacks associated with copper-catalyzed cycloaddition while employing the 2OMePS ASO backbone, resulting in the formation of oligonucleotide-peptide conjugates with high conversion rates and minimal desulfurization.³⁴ Moreover use of Cu (I) stabilizing ligand enhances the reaction rate multiple times while acting as a sacrificial reductant, thereby safeguarding the biomolecules from oxidative stress.³⁵

Overall, our results suggest that the identified lead peptide ETWWK upon conjugation with 2OMePS ASO, exhibits promising potential for facilitating targeted delivery of ASOs with notable cellular uptake and precise nuclear localization observed in both C2C12 and human DMD patient-derived cell lines, validated via fluorescence microscopy and flow cytometry. Also our conjugation strategy affords simple and efficient conjugation of CPP with ASO employing click chemistry. This ETWWK-ASO conjugate with excellent biocompatibility holds an immense potential to eventually emerge as a potential ASO based therapeutic option for DMD.

3.3 Results and Discussion:

3.3.1 Design and Synthesis of Cell penetrating peptides

Elevating the bioavailability of ASOs represents a crucial strategy in enhancing the cost-effectiveness and efficacy of ASO-based therapies for DMD. In that regard several ligands having potential for cell penetration has been developed and conjugated with ASO. However, most of these designed CPPs demonstrate significant cell penetration ability, but they are often long and show cytotoxicity at higher concentrations due to the presence of numerous positively charged amino acids such as lysine and arginine residues in the peptide sequence. Additionally, highly cationic charged CPPs like (RXR)₄ and (RXRRBR)₂ are effective in delivering ASO but suffer from off-target localization to the liver and kidneys. Conversely,

muscle-specific peptide ASSLNIAIX exhibits excellent muscle specificity but lacks efficient cell penetration compared to (RXR)₄ and (RXRRBR)₂ conjugated ASO.²⁴ While numerous designed CPPs such as YG(R)₉ and Tat, previously demonstrated efficient internalization into the cytosol but were observed in comparatively reduced quantities within the nuclear compartment.³⁶

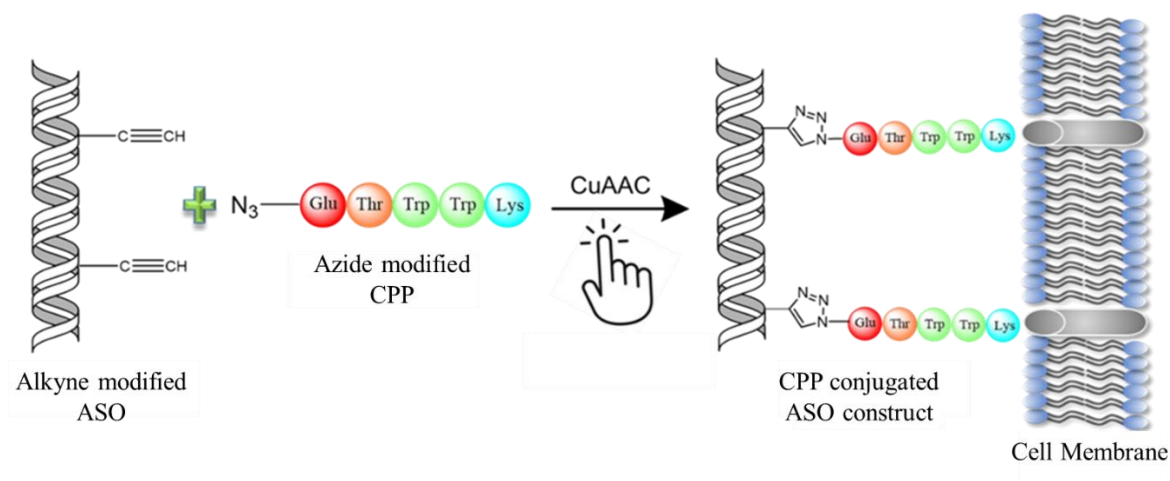


Figure 3.1: Schematic representation of ETWWK-ASO construct synthesis and its further application in cellular internalization.

Herein, we report the design and synthesis of short non cationic 5-mer CPP demonstrating excellent cell penetration as well as nuclear localization ability, which is inspired from the role of two tryptophan moiety successively in mediating both processes via an endocytic pathway. Our prior research has confirmed that the consecutive presence of a dual tryptophan moiety substantially enhances the cell-penetrating capability of the chariot peptide-derived tetrapeptide "ETWW".²⁶ Motivated by our earlier observation here in this study we are interested to further increase the potential of this tetrapeptide for the intercellular delivery of therapeutic macromolecules like ASOs. Though ETWW was found to be a strong CPP candidate but probably the absence of positively charged amino acids like Lysine or Arginine at its C-terminus made its endosomal escape bit difficult and exhibited a lower therapeutic effectivity when tagged with a macromolecule like ASOs.³⁷⁻³⁸ To surpass this impairment initially we have chosen a HIV-TAT₄₉₋₅₇ truncated positively charged tripeptide "KKR" to attach at the C-terminus of the ETWW.³⁹ However, the cytotoxicity profiling of ETWWKKR showed a lower cellular viability. In order to deal this, we have introduced individual single lysine/arginine at the C-termini of it and compared the cell penetrating ability of the modified CPPs with the former.⁴⁰⁻⁴¹ Interestingly both cell-penetrating and cytocompatibility data (Figure 3.30) confirmed the fact that the mere inclusion of a single lysine residue in the CPP sequence substantially enhances cell-penetrating ability without compromising cytotoxicity, particularly within a shorter span of time compared to ETWW. To validate this observation, we assessed the cellular uptake efficiency after a 30-minute of incubation period. Our findings reveals that ETWWK demonstrates a pronounced capability to localize within the nucleus after 30 minutes of treatment, whereas ETWW primarily localizes near the close proximity of the nucleus but not within it, after the same 30 min of incubation in the C2C12 myoblast cell

line, as evidenced by fluorescence microscopy techniques (Figure 3.2). However, after 4 hours of incubation, both ETWWK and ETWW shows equal localization within the nucleus.

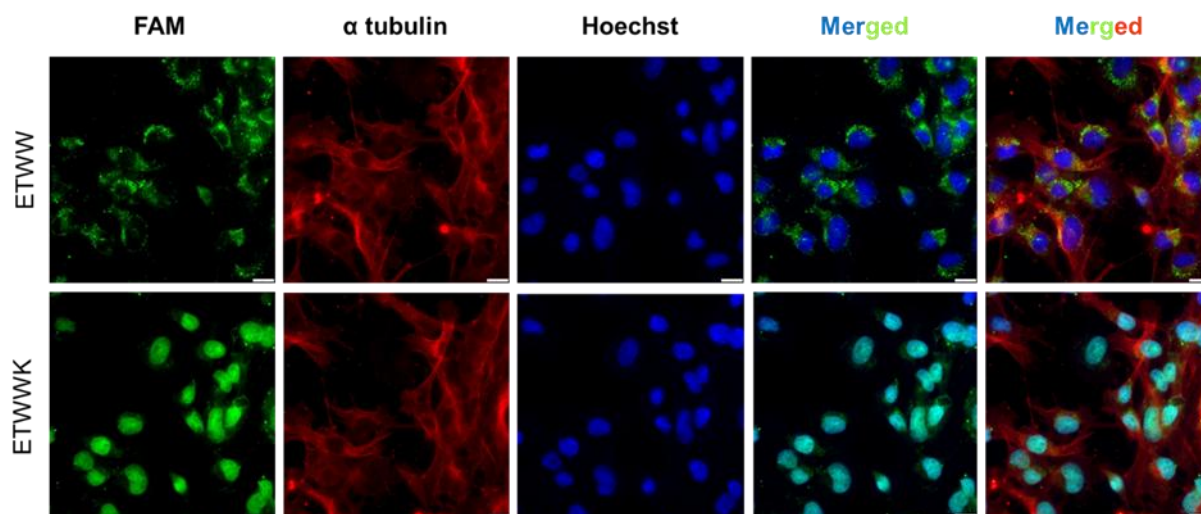


Figure 3.2: Comparative cellular uptake study of FAM labelled ETWW and ETWWK upon treatment with C2C12 cells after 30 minutes of incubation at a test concentration of 5 μ M. Upper panel with ETWW shows presence of FAM tagged CPP at close proximity to the nuclear compartment whereas lower panel shows presence of FAM labelled ETWWK inside the nuclear compartment. Images were captured through fluorescence microscope at FITC, TRITC and DAPI channel. Scale bar corresponds to 20 μ m.

This expedited nuclear delivery is advantageous for CPP-ASO therapeutics, as peptides often face compromised serum stability over exposure to prolonged durations. Thus, any CPP stretch capable of delivering cargo into the nucleus more rapidly at the site of splicing will achieve the ultimate goal of decreasing the dosing frequency of ASO based therapeutics. Moreover, we observed that the inclusion of a single arginine residue led to compromised cell viability, prompting its exclusion from the list of lead CPP candidates for cellular uptake studies. We anticipate that ETWWR would also exhibit similar potency as of ETWWK in delivering cargo ASO. However, considering the cytotoxic effects associated with arginine, as evidenced by our MTT data (Figure 3.38) and supported by existing literature,⁴²⁻⁴⁴ we selected ETWWK as the lead CPP candidate for investigating cellular uptake. In this regard ETWWK peptide impart superior therapeutic activity to deliver ASO precisely to nucleus with excellent cytocompatibility.

The CPP-ASO conjugated product exhibits substantial cellular uptake with precise nuclear localization compared to unconjugated ASO, as validated preliminarily by immunocytochemistry and flow cytometry. The selected short 5-mer lead peptide “ETWWK”, chosen from a set of four CPPs, was subsequently conjugated with 2OMePS ASO utilizing click chemistry, targeting exon 51 of the dystrophin gene. To further assess the conjugation efficiency of the CPP-ASO conjugation technique under different condition, all four designed peptides (ETWW, ETWWK, ETWWR, and ETWWKKR) were conjugated with ASO under both in presence of ligand and in ligand free condition, as discussed later.

3.3.2 Conjugation of azide functionalized CPPs with alkyne containing ASO and vice versa

Prior to the synthesis of active alkyne linker and its attachment to the ASO, and similarly N-terminal amine to azide conversion, several other strategy was explored for conjugating CPPs with ASO. Initially, ASO was functionalized with an azide fragment by attachment of ASO with azidoacetic acid (Figure 3.3) and simultaneously peptide was functionalized with an alkyne moiety by attachment of pentynoic acid (Figure 3.5). Whereas in another approach functionalization of ASO with alkyne group was attempted by conjugation of ASO with pentynoic acid (Figure 3.4) as pendant arms of this acid is long enough to reach the site of action⁴⁵ and subsequently CPP was functionalized with an azide fragment, for which (CPP-1) ETWW was attached with azidoacetic acid (Figure 3.6). Next coupling reaction were performed using CuI and CuBr.Me₂S both, however we could not achieve conjugation likely because of the inactive alkyne fragment, which ultimately leads to unproductive complexation of copper with the alkyne fragment, which is an essential prerequisite for click chemistry. Also, sometimes alkynoic acid derivatives undergo competing intramolecular cyclization to form enol lactones under conditions typically applied for click chemistry, resulting in lower yields of the desired triazole product.⁴⁶ Altogether we cannot achieve conjugation employing this strategy. Mass spectra of the building blocks are as follows. (Figure 3.3-3.6).

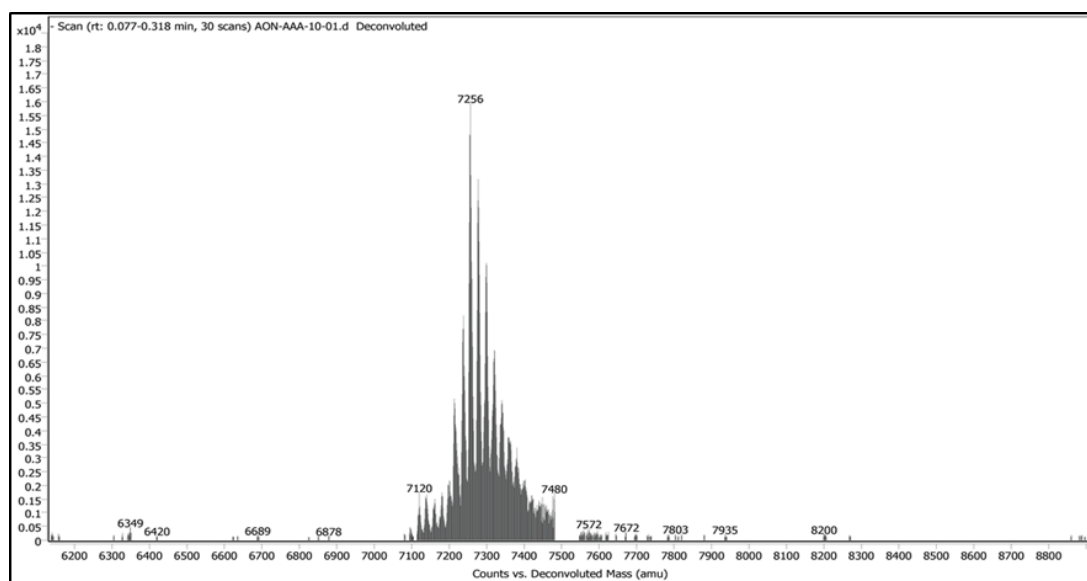


Figure 3.3: Deconvoluted Mass spectra of ASO attached with azidoacetic acid

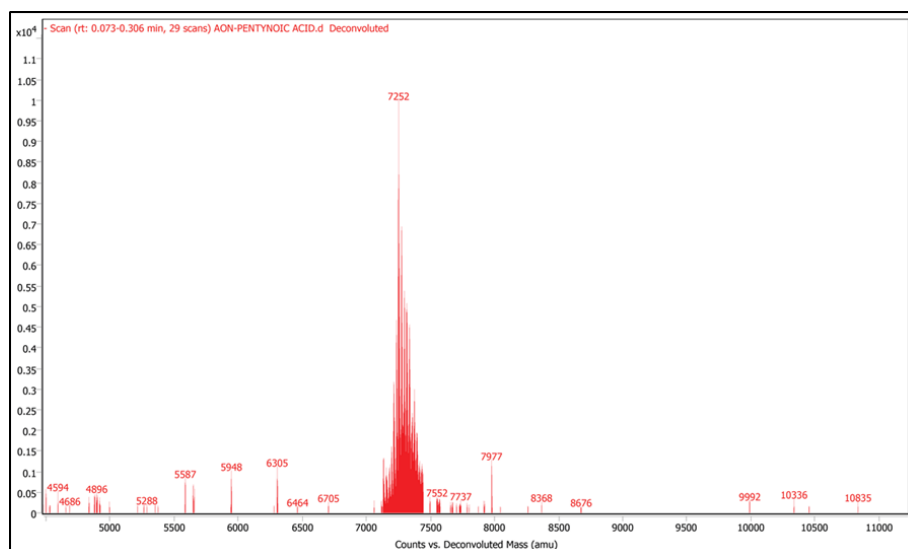


Figure 3.4: Deconvoluted Mass spectra of ASO attached with pentynoic acid.

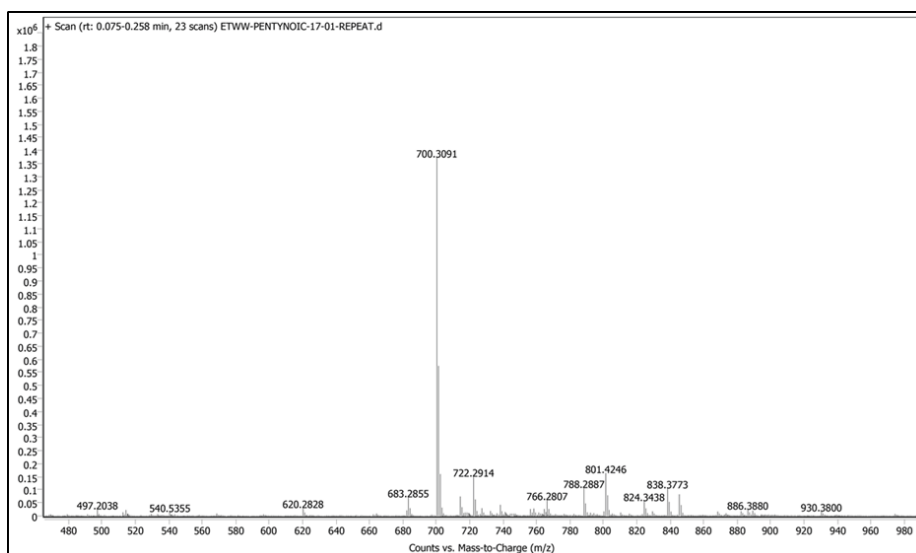


Figure 3.5: Mass spectra of CPP-1 (ETWW) attached with pentynoic acid

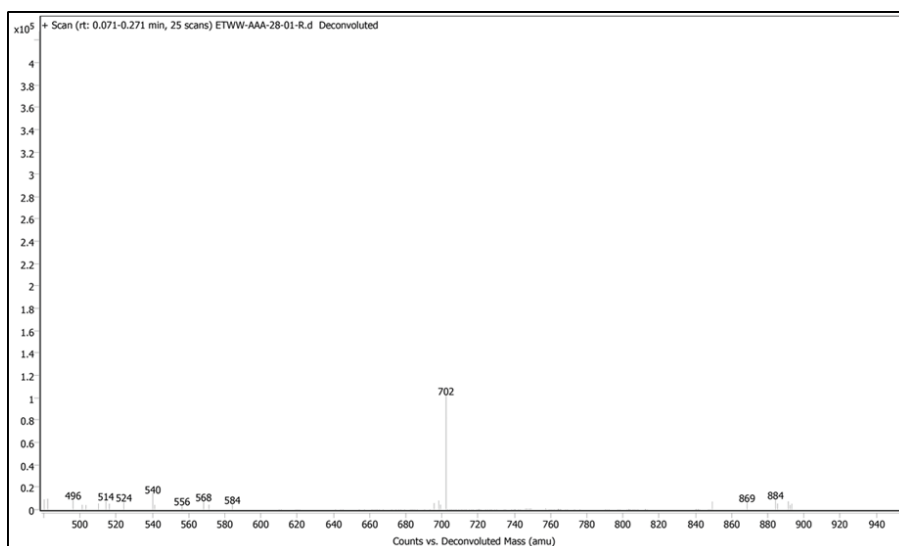


Figure 3.6: Mass spectra of CPP-1 (ETWW) attached with azidoacetic acid

3.3.3 Conjugation of N-terminal azide functionalized CPPs with active alkyne containing ASO:

Conjugation of azide functionalized CPPs were conjugated with alkyne functionalized 2OMePS ASO employing click chemistry. Different coupling strategies via click chemistry were performed both in presence and absence of Cu(I) stabilizing ligand.

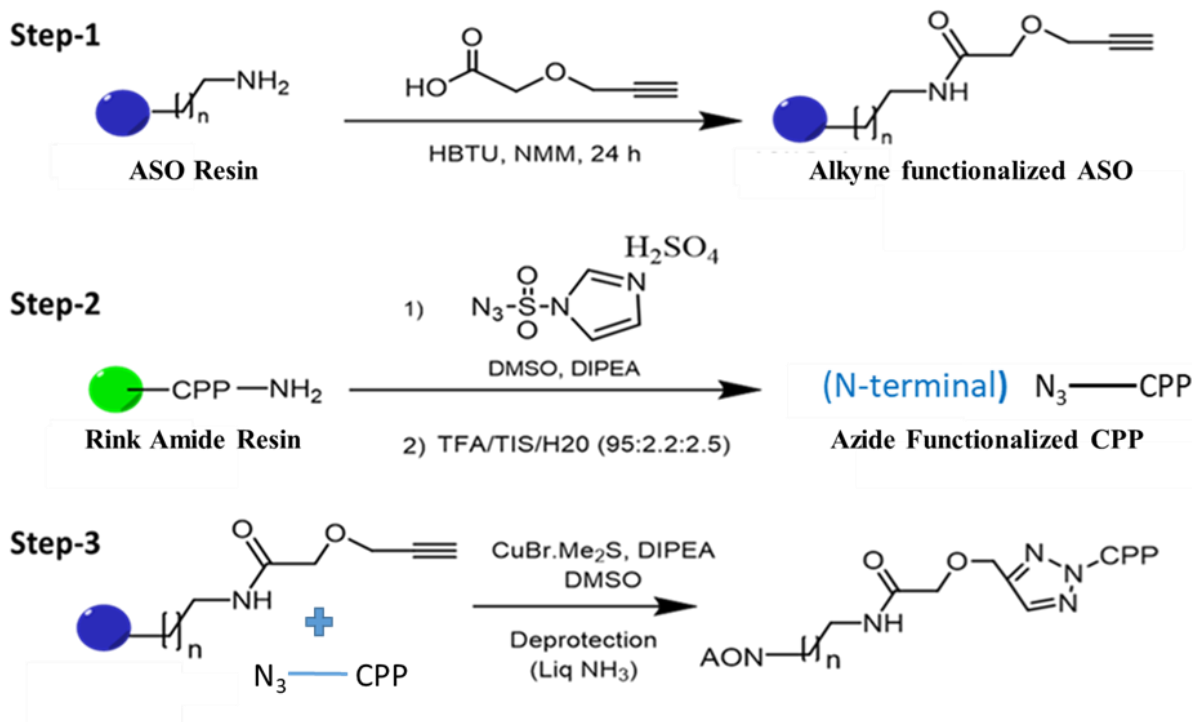


Figure 3.7: Schematic representation for CPP-ASO coupling via click chemistry. Lab synthesized active alkyne motif was attached with ASO (Step-1), and simultaneously N-terminal amine was converted to azide via azo

transfer reagent (Step-2). In step-3, the azide containing CPPs was conjugated to alkyne containing ASO via click chemistry.

Herein after trial with Copper iodide (CuI), which shows higher extent of desulfurization product we shifted our focus for employing CuBr.Me₂S as choice of Cu(I). CuI was avoided as a catalyst due to its susceptibility to induce significant desulfurization. To identify the optimal conditions for click reaction on the solid support of PS-based ASO with minimal desulfurization and maximum conjugation, CuBr.Me₂S was chosen as the Cu(I) source. The selection of CuBr.Me₂S, a complex salt where copper is already coordinated to a sulfur ligand, suggests a diminished susceptibility of PS backbone-based ASO to desulfurization.³⁴ In this study, we have developed a tailored conjugation method to facilitate the efficient and straightforward coupling of CPPs with the 2OMePS ASO backbone using click chemistry, as shown in Figure 3.7. To overshoot the challenges encountered during the coupling of CPPs to ASO as discussed under section 3.2.2, we synthesized an alkyne containing linker (SG-B-59). Characterization data of the linker (Figure 3.10-3.13). This linker was subsequently attached to the ASO for its modification with an active alkyne motif (Figure 3.8). This newly synthesized linker features an adjacent oxygen atom, thereby enhancing the reactivity of the alkyne motif, thus copper (I) ions readily form complex with the alkyne fragment, facilitating the formation of desired triazole product and enabling successful conjugation of CPPs with the ASO under click chemistry conditions employing CuBr.Me₂S as catalyst.

On the other hand, while encountering setbacks utilizing ETWW conjugated to azidoacetic acid, we redirected our focus to an alternative approach. Herein, we directly converted the N-terminal amine to azide using the synthesized imidazole-1-sulfonyl azide hydrogen sulfate (ISA.H₂SO₄) in solid phase, employing rink amide resin.^{33, 47-48} Characterization data of the synthesized azo transfer reagent ISA.H₂SO₄ (Figure 3.14-3.16). Alternatively, side-chain modification of lysine residues to introduce an azide group is also an effective strategy for conjugation. Researchers may opt for side-chain modification if a lysine residue is present within the peptide sequence.

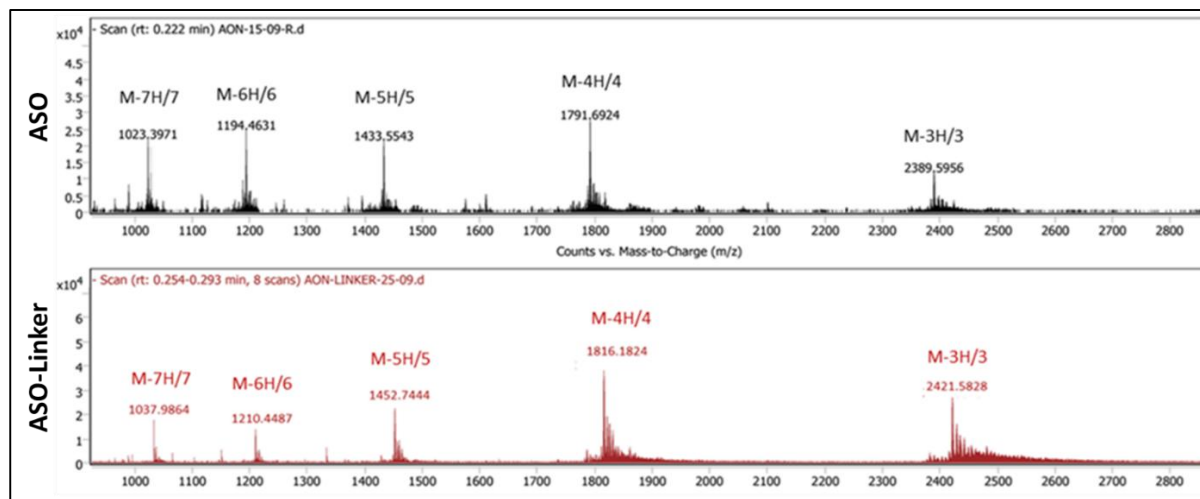


Figure 3.8: Comparative mass spectra of ASO and ASO attached with linker. Mass peaks observed at M-3H/3, M-4H/4, M-5H/5, M-6H/6, and M-7 H/7.

In the absence of side-chain amine-containing amino acids, modification of the N-terminal amine to an azide remains a viable approach for conjugation. This newly modified N-terminal azide functionalized CPP not only facilitated successful coupling with the ASO but also reduced the cost of the overall conjugation process by avoiding the need for attaching expensive azide linker to the CPPs. Next, different click chemistry strategy was employed, both in the presence and absence of Cu(I) stabilizing ligand, utilizing CuBr.Me₂S as a source of copper catalyst. Additionally, coupling strategy was also performed in the presence of sodium ascorbate/ CuSO₄ pair. Altogether, different coupling conditions were employed to couple CPP with ASO utilizing click chemistry in solid phase, which provide advantage of removing excess copper through EDTA wash, where conjugation success was dependent on the amino acid sequence of the CPP.

Whereas, in standard condition while performing click chemistry for CPP-ASO conjugation utilizing Cu(I) catalysis is known to degrade ASO due to the presence of hydroxyl radicals. Thus, Cu(I) stabilizing ligands are often necessary to prevent Cu(I) from oxidation and disproportionation. By employing a Cu(I)-stabilizing ligand, not only Cu(I) disproportionation is inhibited, while the excess ligand also serves as a sacrificial ROS scavenger, further protecting biomolecules from oxidative damage.⁴⁹ The ligand not only accelerates the reaction many folds but also functions as a sacrificial reductant, minimizing the risk of oxidation.³⁵ Additionally, ligands binds and sequester Cu(I) ions, thus blocking the bioavailability of Cu(I) and mitigating potential toxicity, and preventing bio molecular damage while maintaining catalytic efficiency in click conjugation.⁴⁹

In this study, we focused on C_{3v}-symmetric tripodal ligands such as polytriazoles and polybenzimidazole which is often used to mitigate the DNA degradation and also concurrently accelerate the copper catalyzed azide alkyne cycloaddition (CuAAC) process.⁵⁰ The choice of tripodal ligands was further motivated by their ability to provide a cleaner metal coordination site.⁴⁵ In this study, we used TBTA (tris-benzyl triazole motif) and (BimH)₃ (tris-benzimidazole motif) as Cu(I) stabilizing ligand, both of which are soluble in DMSO. Herein, choice of DMSO for click chemistry was considered as it serves as a known radical scavenger which effectively suppressing oligonucleotide damage during the click reaction.^{49, 51} Next in consideration of click chemistry, water was used in conjunction with strong coordinating solvent DMSO to ensure biocompatibility, owing to the high aqueous solubility of most cupric compounds.^{49, 52} Consequently, a mixture of DMSO and water in a volumetric ratio of 9:1 was selected for the experimental procedure. In our approach choice of 1:1 Cu/Ligand were focused as the active catalyst likely to involve 1:1 copper to ligand combination for bio conjugation application.⁴⁵ Our observation revealed that both (BimH)₃ and TBTA facilitate better conversion to conjugated triazole product in comparison to ligand free techniques.^{45, 53}

However, among the two, (BimH)₃ comparatively yielded better results than TBTA. Comparative yield conversion determination via HPLC are detailed in Table 3.1. This disparity between two ligands may be attributed to their distinct mode of binding to copper in 1:1 ligand to copper ratio either in a productive or inhibitory manner, in the presence of strong coordinating solvent DMSO.

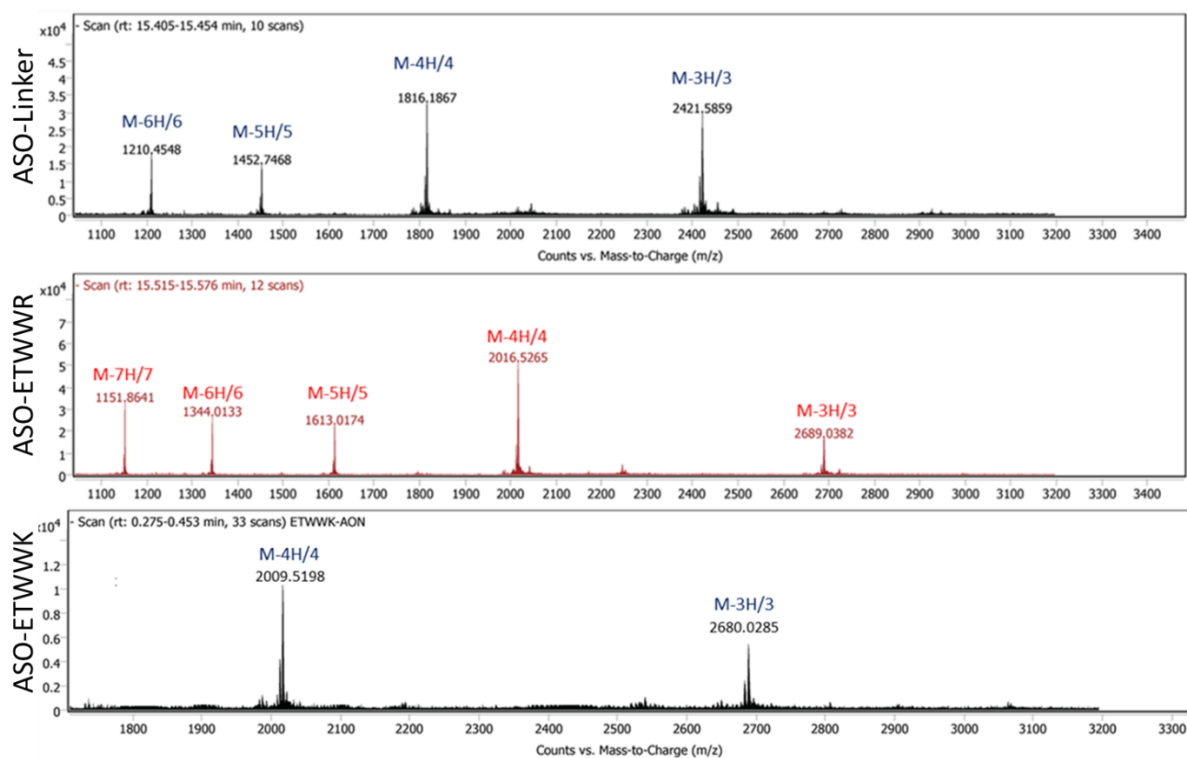


Figure 3.9: Comparative mass spectra of ASO-linker, ETWWR conjugated ASO, and ETWWK conjugated ASO.

Likely TBTA leads to form inhibitory complex with copper in the presence of strong donor coordinating solvent DMSO when 1:1 ligand to copper ratio was used, as TBTA containing poly triazole motif are considered a weak binders to copper and thus DMSO can compete with this ligands for Cu binding sites and thereby diminishing ligand based acceleration. Whereas, (BimH)₃ containing benzimidazole motif are considered to be strong binders with copper and can replace strong donor solvent DMSO and thus leads to productive Cu binding and facilitates ligand accelerated catalysis. Overall, in strong donor environment such as with DMSO ligands with stronger donor arms such as polybenzimidazoles are preferred over poly triazoles to promote the formation of catalytically active complexes involving two copper centers.^{45, 50}

Whereas the presence of positively charged amino acid arginine within the CPP obviates the need for additional Cu (I) stabilizing ligand, as imine nitrogen of arginine side chain efficiently stabilize Cu(I). Arginine within the CPP performs a similar function to Cu(I) binding ligands contributing to the stabilization of Cu(I).⁵⁴⁻⁵⁵ Consequently, ligand-free techniques utilizing CuBr.Me₂S alone were adopted for CPPs containing arginine.³⁴ Comparative mass spectra of ASO attached with linker and its subsequent attachment with CPPs via click chemistry is illustrated in Figure 3.9 and others from figures 3.19 to 3.28.

In conclusion, ligand-accelerated click chemistry condition were identified as optimal for the conjugation of CPPs with ASO. Whereas, the conjugation of CPPs containing arginine within the sequence facilitates conjugation with ASO under ligand free condition in the presence of CuSO₄ and sodium ascorbate pair which yielded moderately better result. Whereas, under

absence of any additives CPP-ASO conjugation resulted in poor yields. Our coupling strategy also assists the coupling of highly positively charged CPP sequence ETWWKKR containing more than two positive charged amino acid to couple with negatively charged ASO. Typically, conjugation of peptides with more than two positively charged amino acids encounters hurdles when coupled to ASO.²⁵

To optimize the reaction conditions, we evaluated the conjugation efficiency of ETWWR with ASO under various conditions. The conjugation was performed using the CuSO₄/sodium ascorbate system under ligand-free conditions and in the presence of ligands such as TBTA and (BimH)₃. ETWWR was chosen for optimization due to the presence of arginine residues, whose imine nitrogen effectively stabilizes Cu(I), thereby facilitating the reaction under ligand-free conditions. However, conjugation under ligand-free conditions results only 65% conversion.

S. No	ASO Sequence (5'-3') and CPP-ASO Sequence	Molecular Weight of ASO	%Conversion For click chemistry	Reference Figures (Mass spectra)	Reference Figures (HPLC spectra showing %conversion)
1.	UCA AGG AAG AUG GCA UUU CU (ASO) C₂₁₇H₂₈₉N₇₇O₁₂₁P₂₀S₂₀ (ASO attached with MMT-Amino C-6 CED phosphoramidite linker at 5' end followed by deprotection of the MMT group)	7173(<i>obs.</i>) 7172.79(<i>calc.</i>)		3.8, 3.19	
2.	ASO Attached with Synthesized Alkyne linker	7269(<i>obs.</i>) 7268.87(<i>calc.</i>)		3.8, 3.19	
3.	ETWWR-ASO (Optimized further under different reaction condition, employing ligand free techniques and in presence of two different ligand (BimH) ₃ and TBTA	8071(<i>obs.</i>) 8070.78(<i>calc.</i>)	89% (BimH) ₃ 82% (TBTA) 65 % (Ligand free technique)	3.9, 3.21	3.29 3.30 3.31
4.	ETWWK-ASO	8043(<i>obs.</i>) 8042.85(<i>calc.</i>)	78% (TBTA)	3.9, 3.20	3.32
5.	ETWW-ASO	7915(<i>obs.</i>) 7914.450(<i>calc.</i>)	72 % (TBTA)	3.22-3.23	3.33
6.	ETWWKKR-ASO	8327(<i>obs.</i>) 8326.33(<i>calc.</i>)	85% (TBTA)	3.24-3.25	3.34
7.	ETWWK(FAM)-ASO	8401(<i>obs.</i>) 8400.89(<i>calc.</i>)	81% (TBTA)	3.26-3.27	3.35
8.	ASO-FAM	7531(<i>obs.</i>) 7530.67(<i>calc.</i>)		3.28	

Table 3.1: Sequence of ASO and CPP-ASO conjugates with corresponding molecular weights and percentage conversion of product under varied condition and with different CPPs.

In contrast, the use of ligands significantly improved conjugation efficiency, here (BimH)₃ resulted in an 89% yield, while TBTA achieved 85% conversion. These results, determined via HPLC analysis, highlight the importance of ligands in enhancing the reaction rate and efficiency compared to ligand-free conditions. Interestingly, (BimH)₃ performed better than TBTA, likely because TBTA forms inhibitory copper complexes under the employed reaction conditions. Based on these observations, subsequent conjugations of other CPPs with ASO were performed in the presence of (BimH)₃. Further conjugation of CPPs ETWWKKR, ETWW, and ETWWK with ASO were performed in the presence of (BimH)₃ ligand. ETWWKKR, containing arginine residues, achieved 85% conversion, while ETWW and ETWWK yielded 72% and 78%, respectively. Whereas conjugation of FAM tagged ETWWK peptide with ASO results in 78.9 % conversion. Detailed HPLC results are provided in Table 3.1. HPLC results determining percentage conversion of CPP-ASO conjugate are detailed in Table 3.1. The objective of the study was to conjugate the newly designed CPPs, characterized by precise nuclear localization and efficient cellular uptake with ASO to develop a potential therapeutics in the pursuit of DMD by facilitating the efficient transfer of ASO biomolecule into muscle cells.

¹HNMR, ¹³CNMR, Mass and HPLC of the active alkyne linker synthesized followed by Mass spectra and HPLC purity of the lead CPP (ETWWK), and ETWWK-ASO construct. Mass spectra of all the CPP-ASO conjugated products, lastly HPLC spectra showing percentage conversion are as follows.

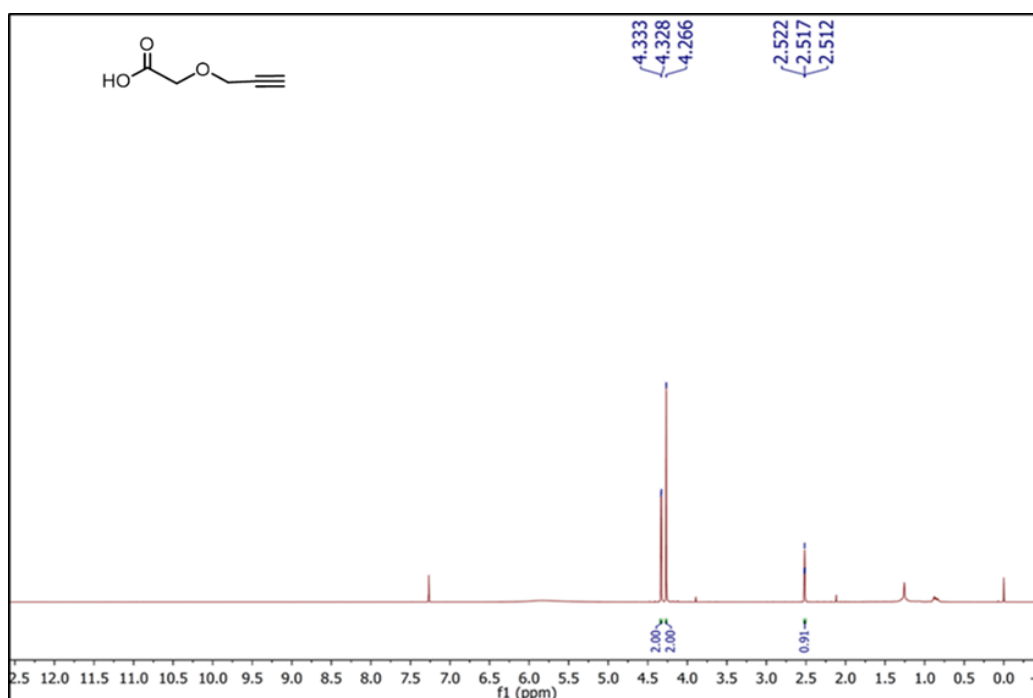


Figure 3.10: ¹H-NMR spectra of synthesized alkyne linker (500 MHz, CDCl₃)

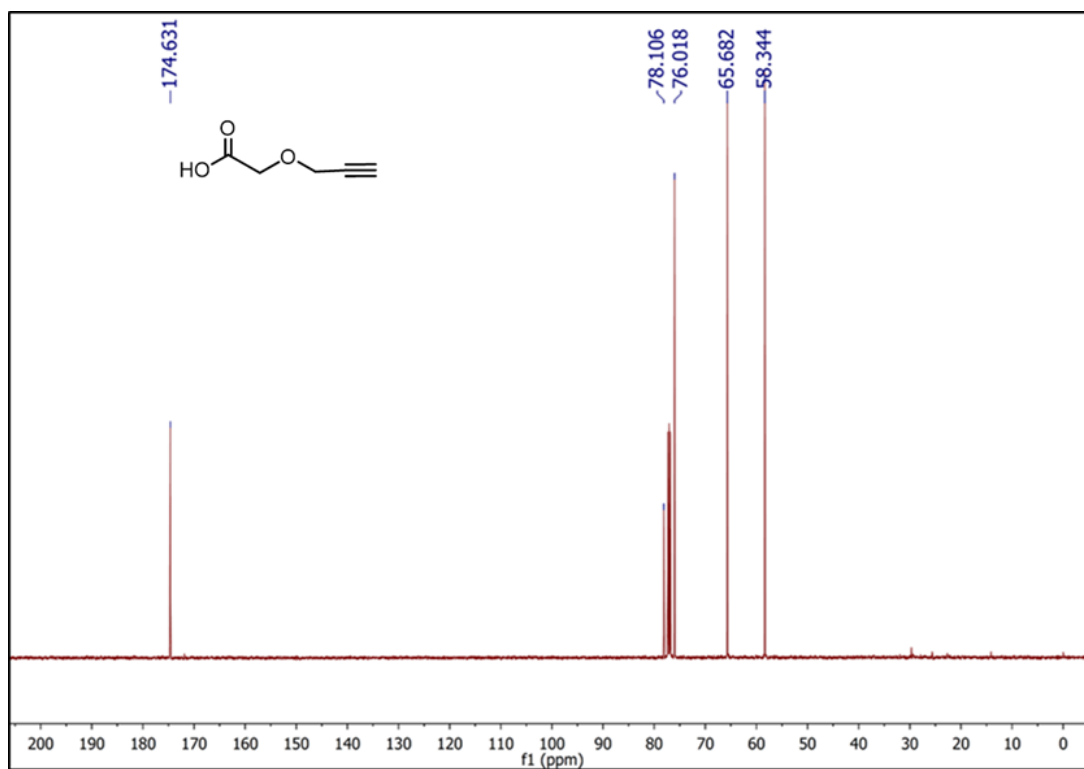


Figure 3.11: ¹³C-NMR spectra of synthesized alkyne linker (125 MHz, CDCL₃)

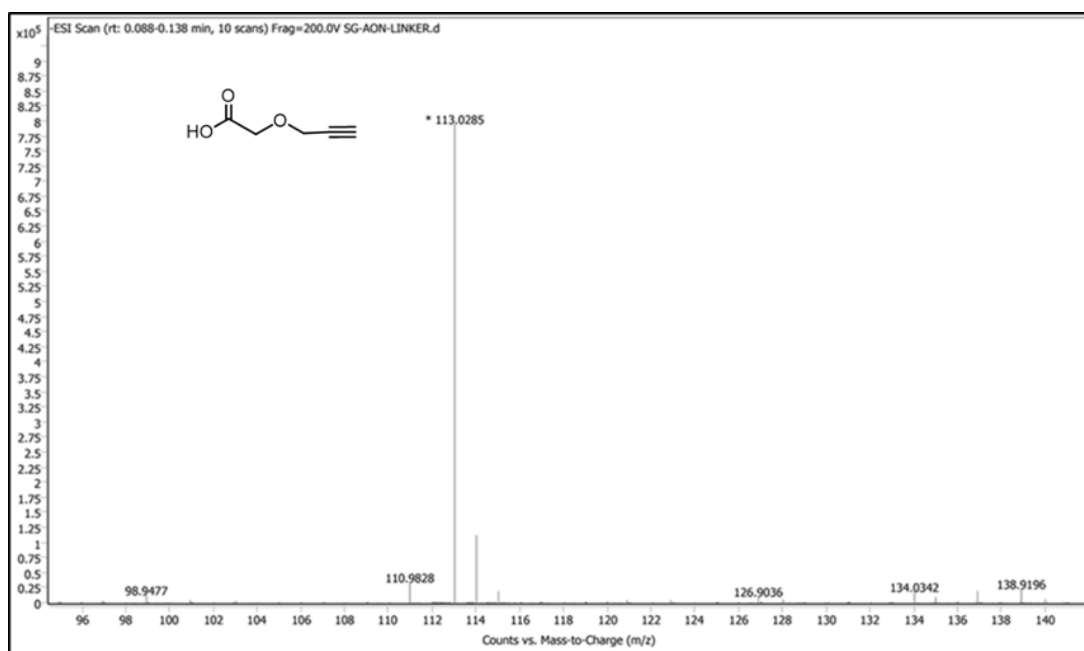


Figure 3.12: Mass spectra of synthesized alkyne linker.

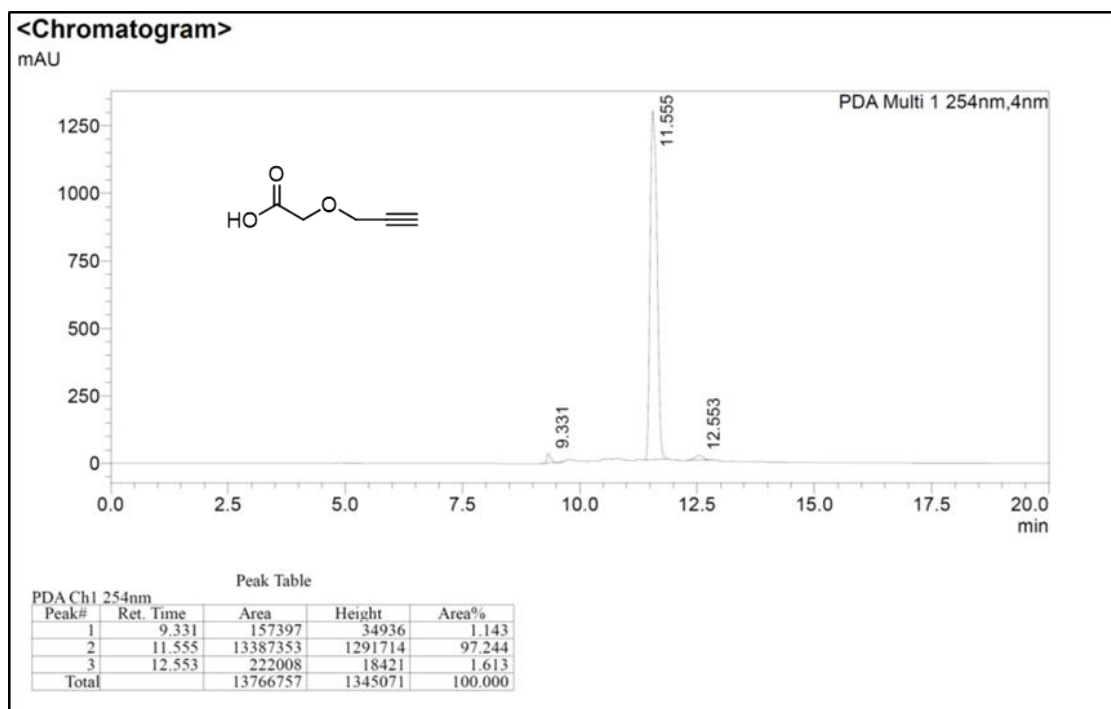


Figure 3.13: HPLC chromatogram of synthesized alkyne linker.

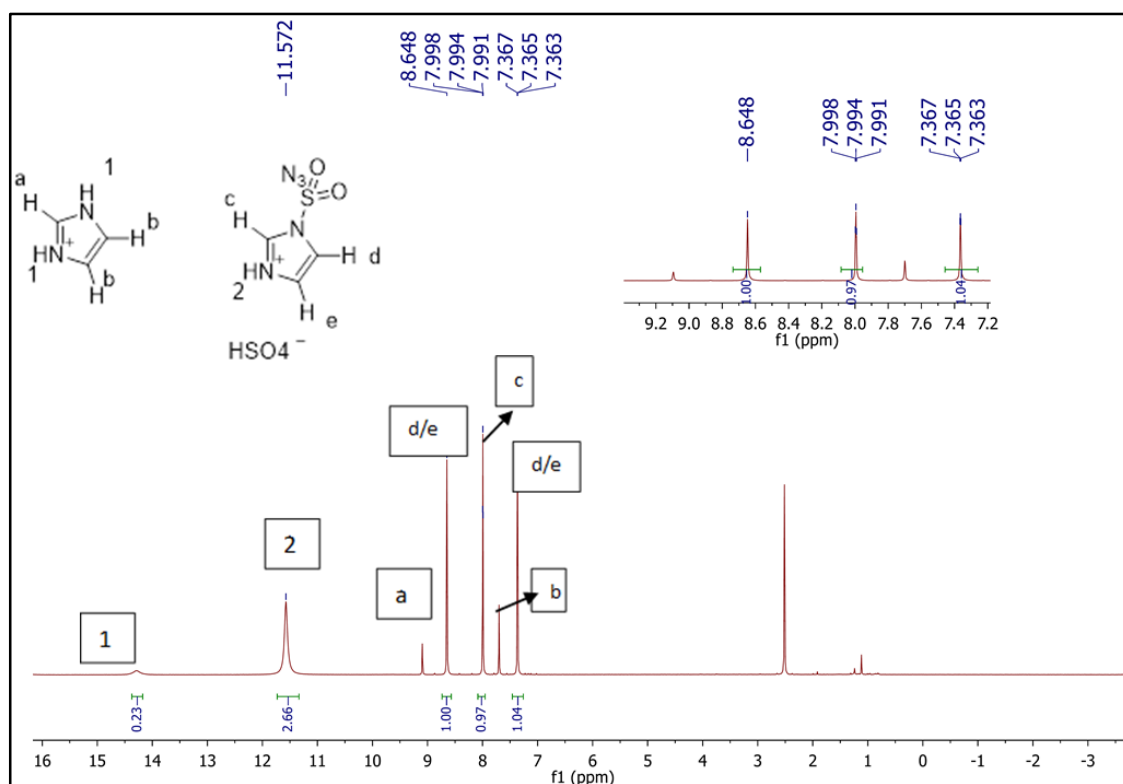


Figure 3.14: ^1H -NMR spectra of synthesized azo transfer reagent (500 MHz, DMSO-d_6)

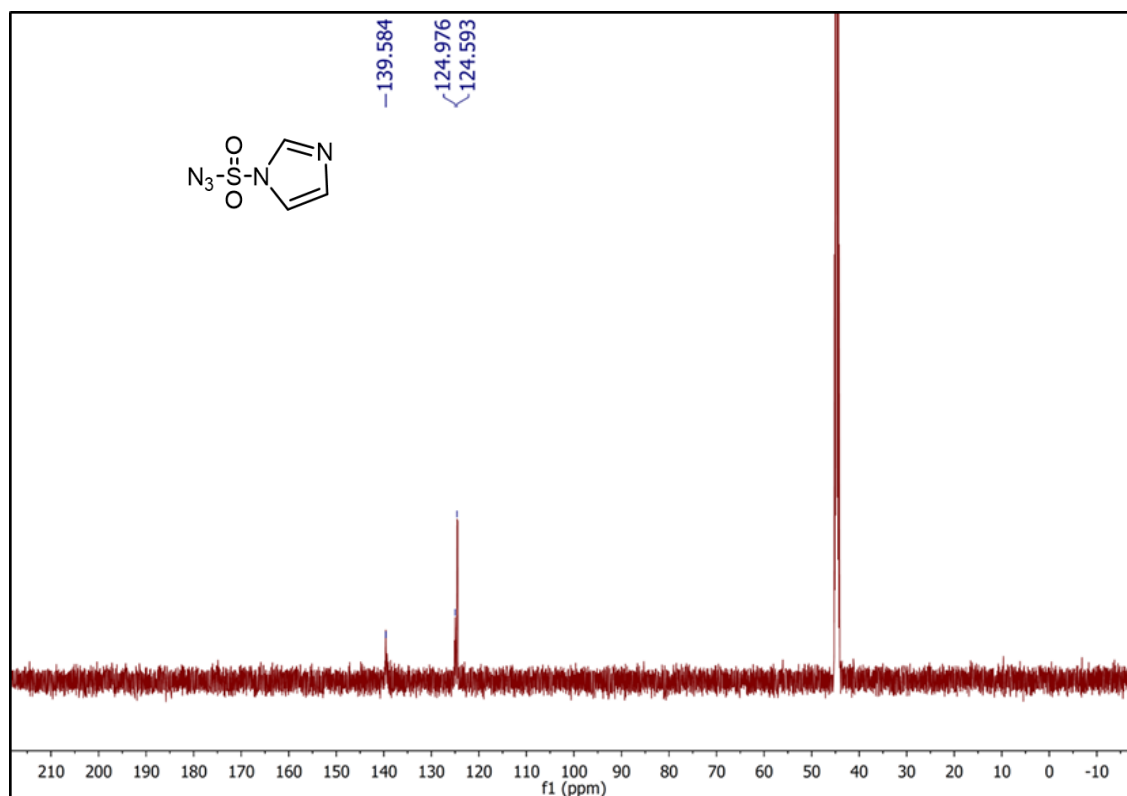


Figure 3.15: ^{13}C -NMR spectra of synthesized azo transfer reagent (125 MHz, DMSO- d_6).

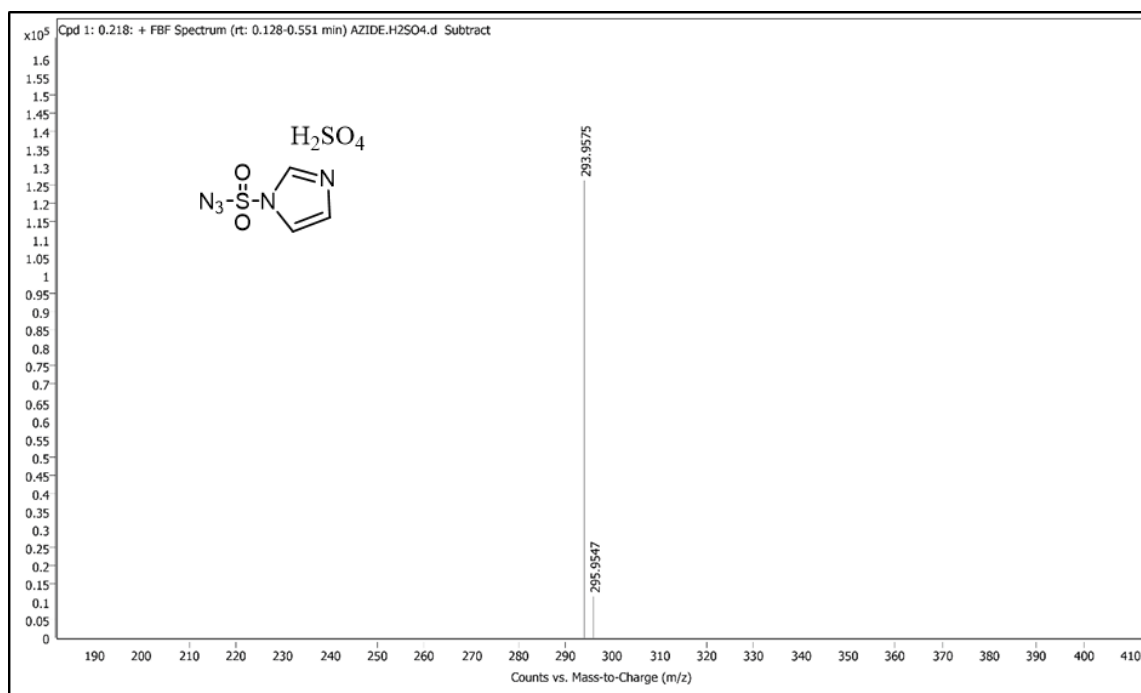


Figure 3.16: Mass spectra of synthesized azo transfer reagent. H_2SO_4 salt

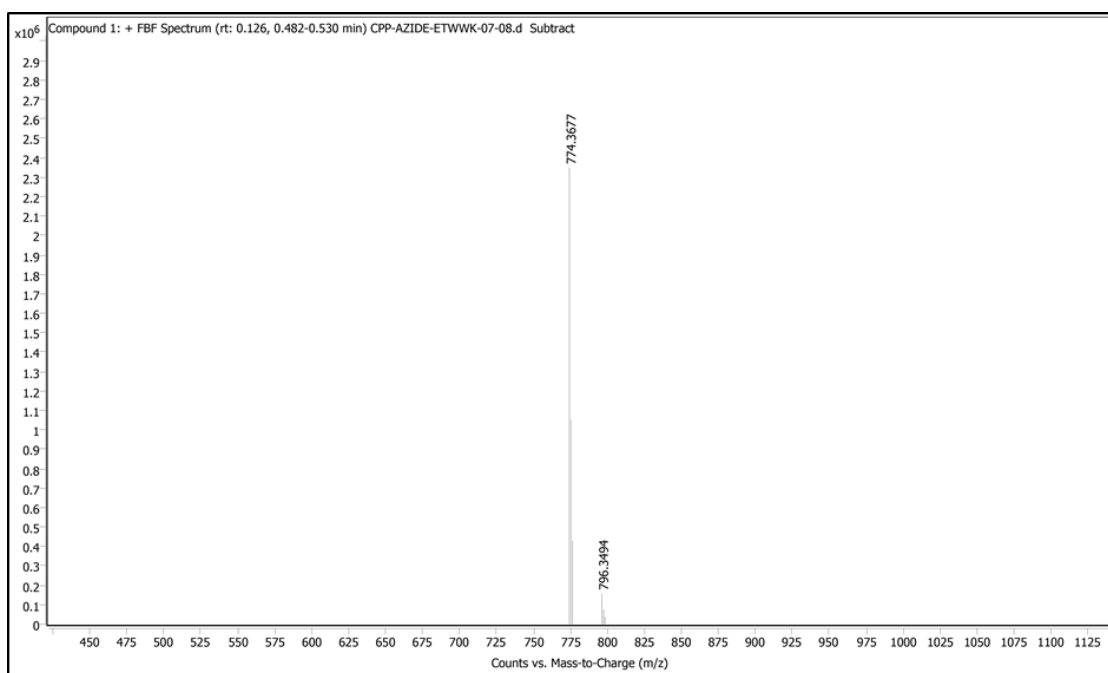


Figure 3.17: Mass spectra of CPP-2 (ETWWK-Azide)

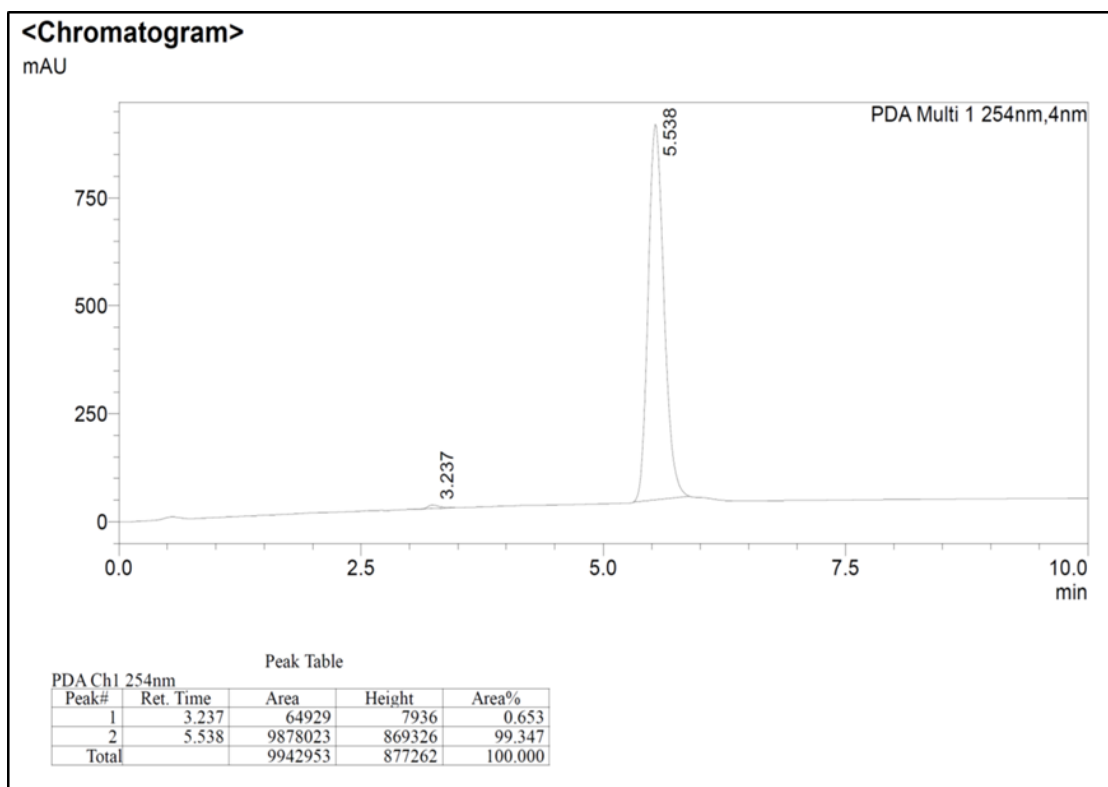


Figure 3.18: HPLC chromatogram of CPP-2 (ETWWK-AZIDE)

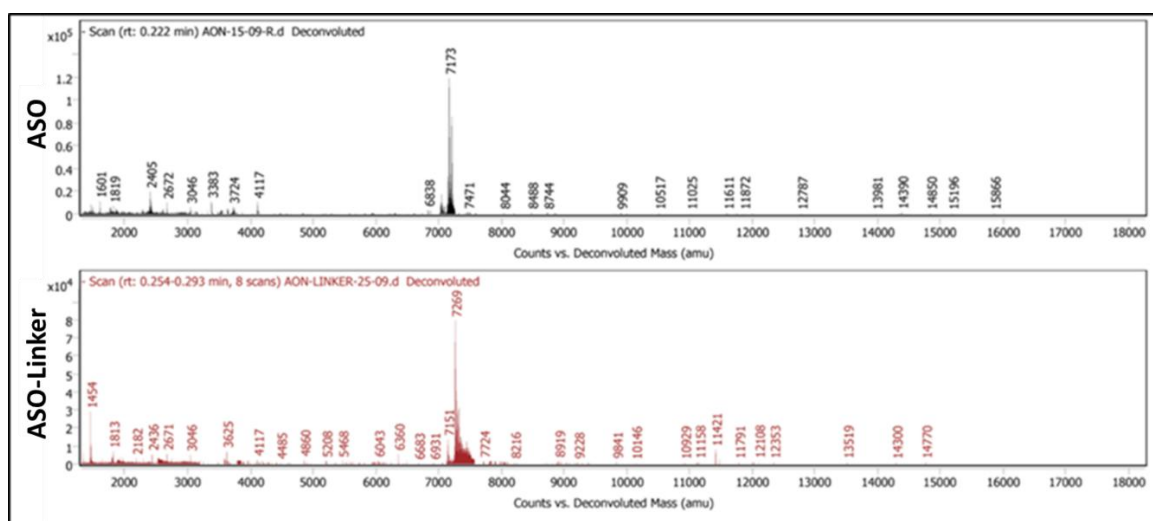


Figure 3.19: Comparative deconvoluted mass spectra between ASO and ASO attached with Alkyne Linker.

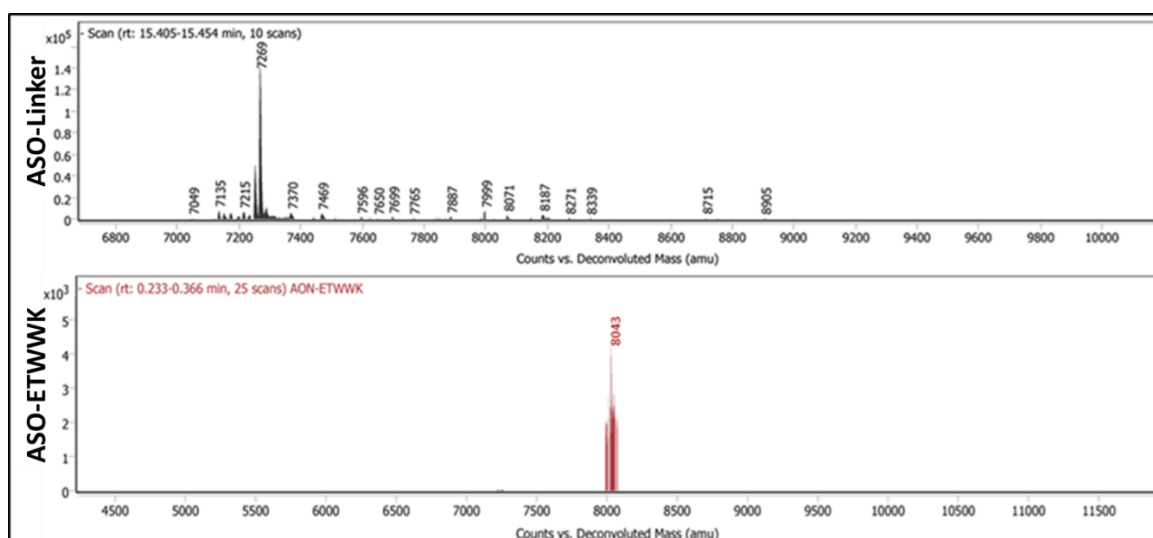


Figure 3.20: Comparative deconvoluted mass spectra between ASO-Linker and ASO attached with ETWWK.

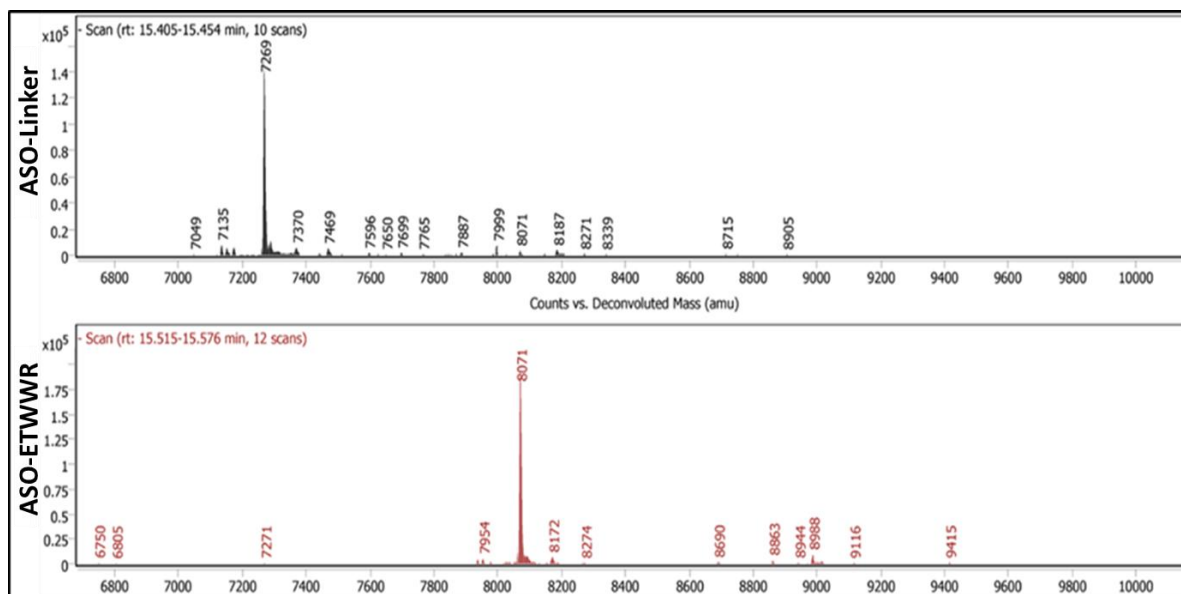


Figure 3.21: Comparative deconvoluted mass spectra between ASO-Linker and ASO-ETWWR.

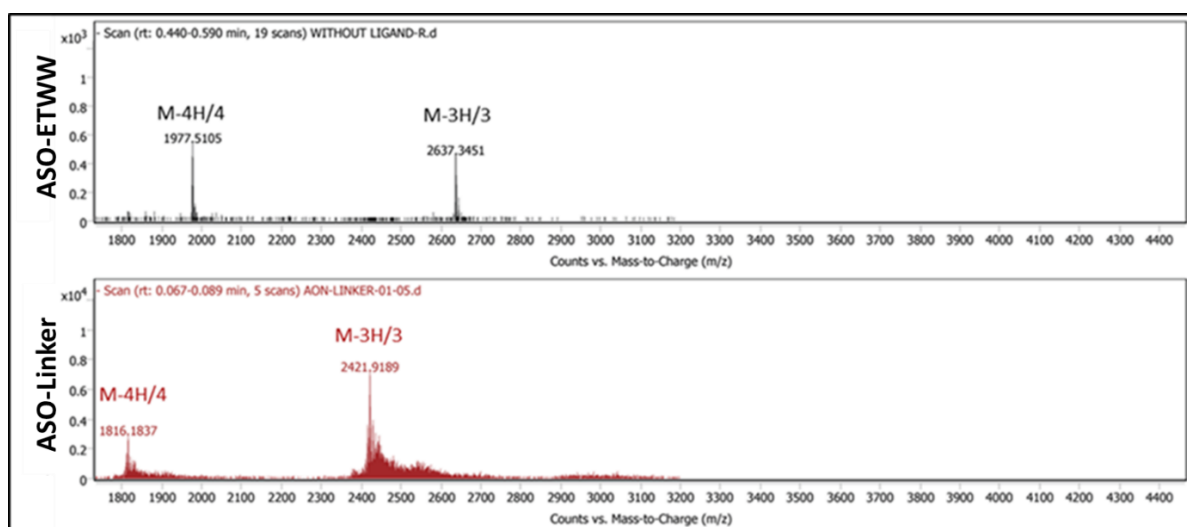


Figure 3.22: Comparative mass spectra between ASO-Linker and ASO attached with ETWW

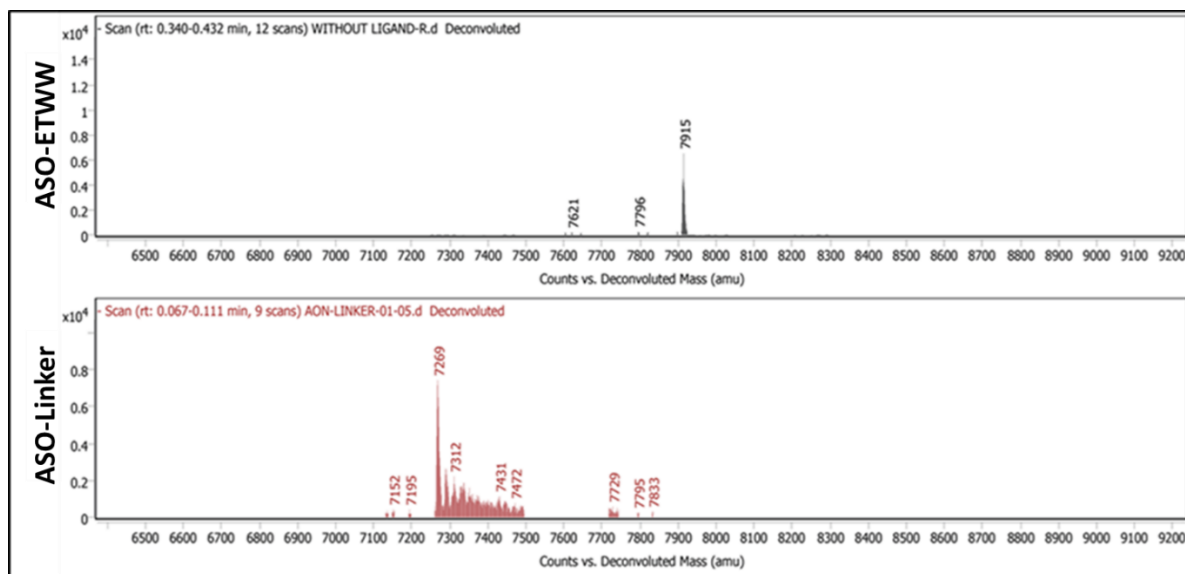


Figure 3.23: Comparative deconvoluted mass spectra between ASO-Linker and ASO-ETWW

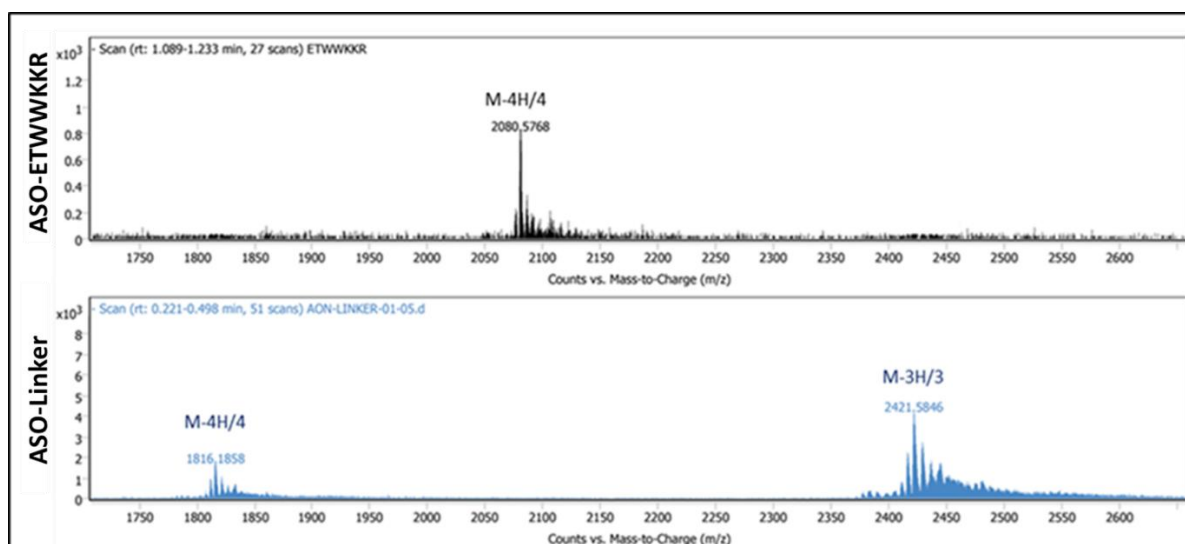


Figure 3.24: Comparative mass spectra between ASO-Linker and ASO attached with ETWWKKR

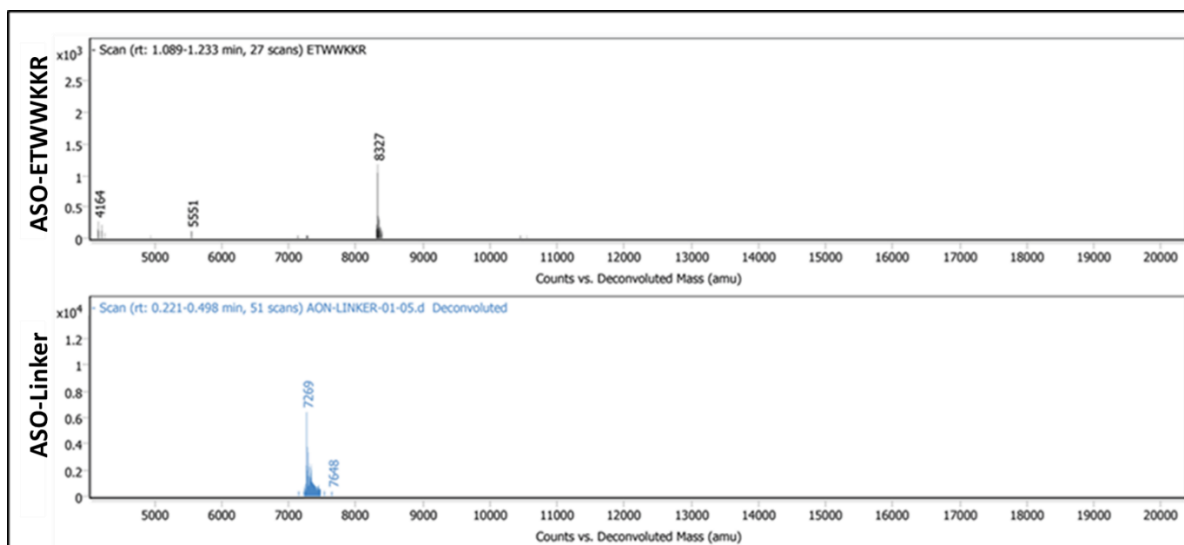


Figure 3.25: Comparative deconvoluted mass spectra between ASO-Linker and ASO-ETWWKKR

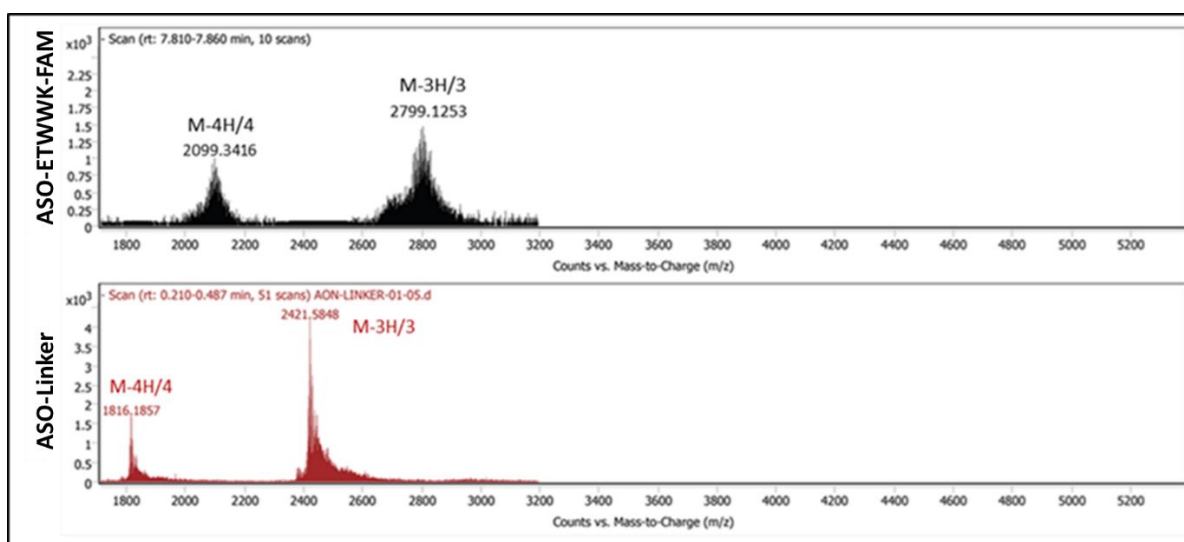


Figure 3.26: Comparative mass spectra between ASO-Linker and ASO attached with ETWWK-FAM

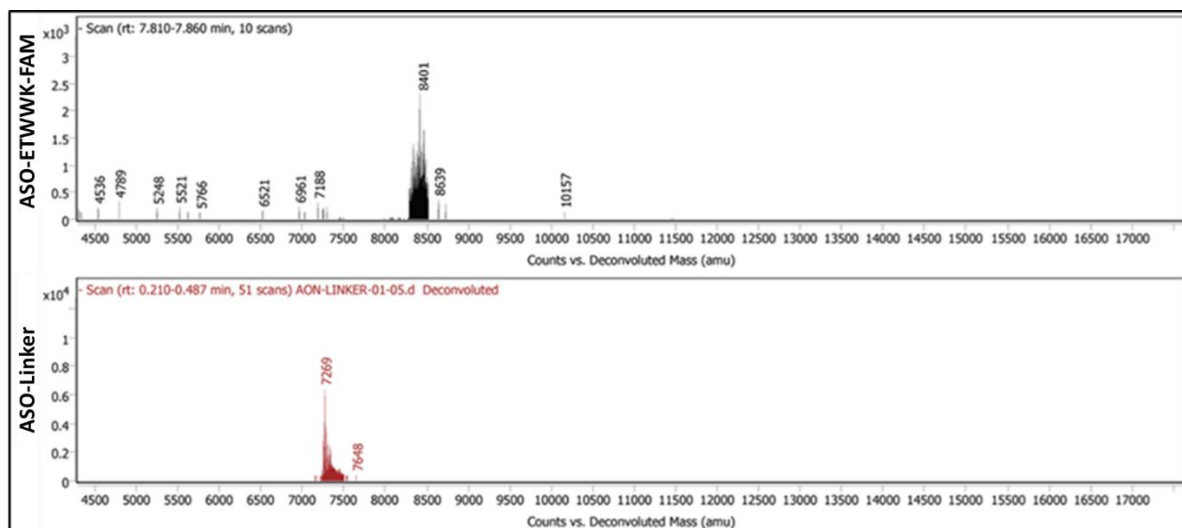


Figure 3.27: Deconvoluted comparative mass spectra of ASO-Linker and ASO-ETWWK-FAM.

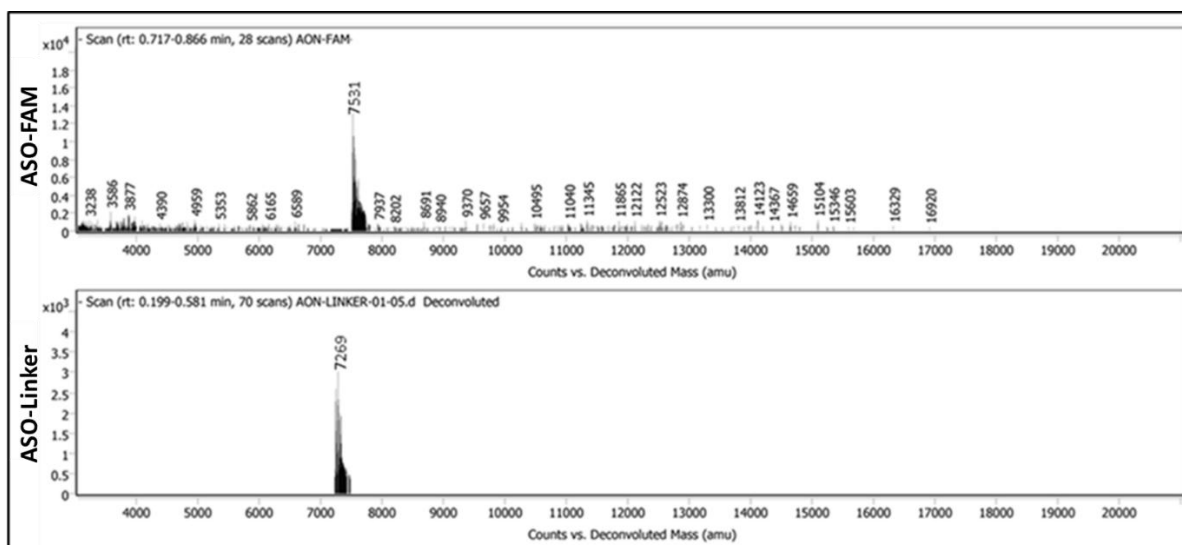


Figure 3.28: Comparative deconvoluted mass spectra of ASO-Linker and ASO-FAM.

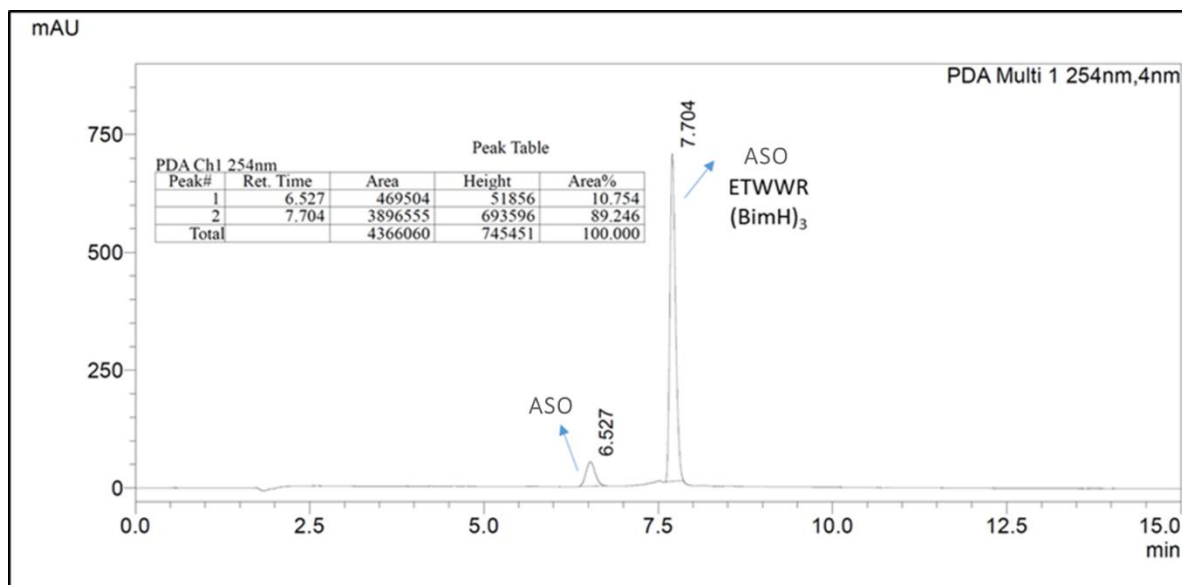


Figure 3.29: HPLC percentage conversion of ASO and ASO-ETWWR with (BimH)₃ ligand

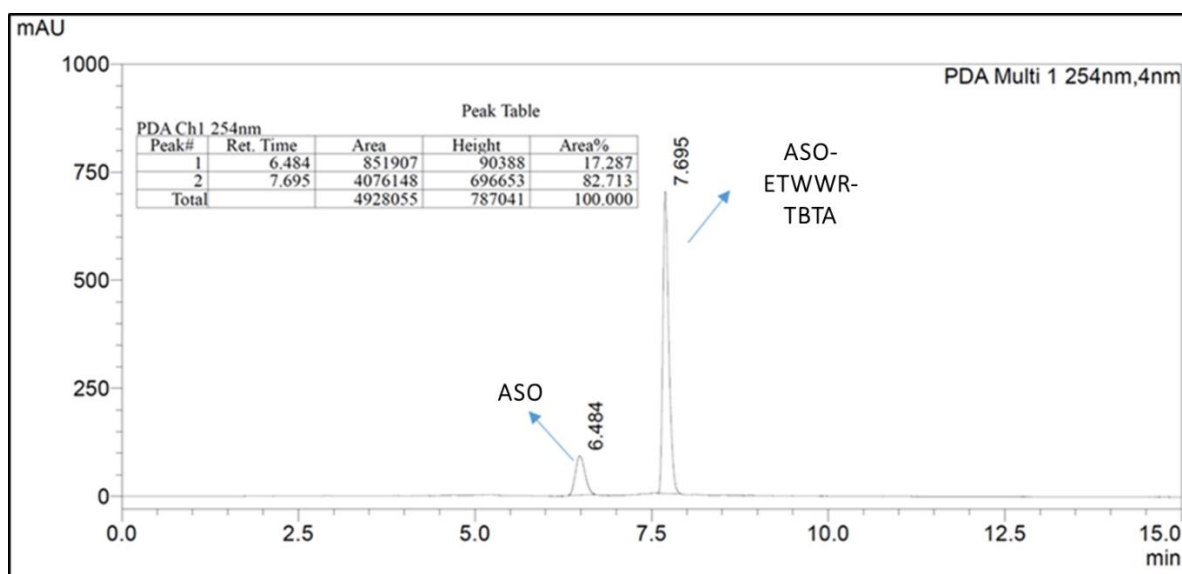


Figure 3.30: HPLC percentage conversion of ASO and ASO-ETWWR with TBTA ligand

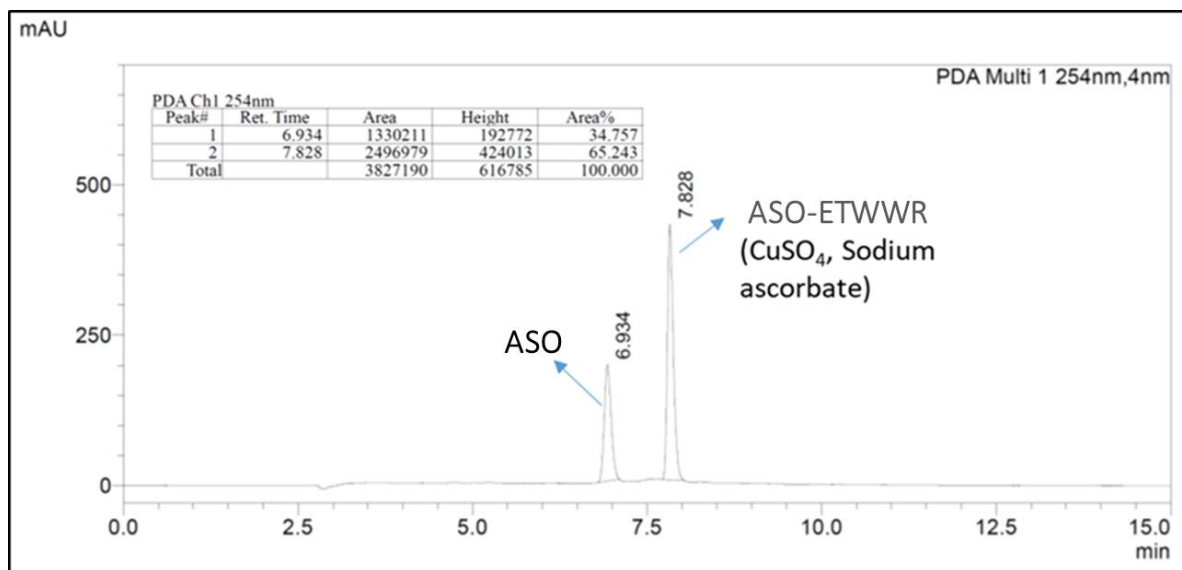


Figure 3.31: HPLC percentage conversion of ASO and ASO-ETWWR under ligand free condition

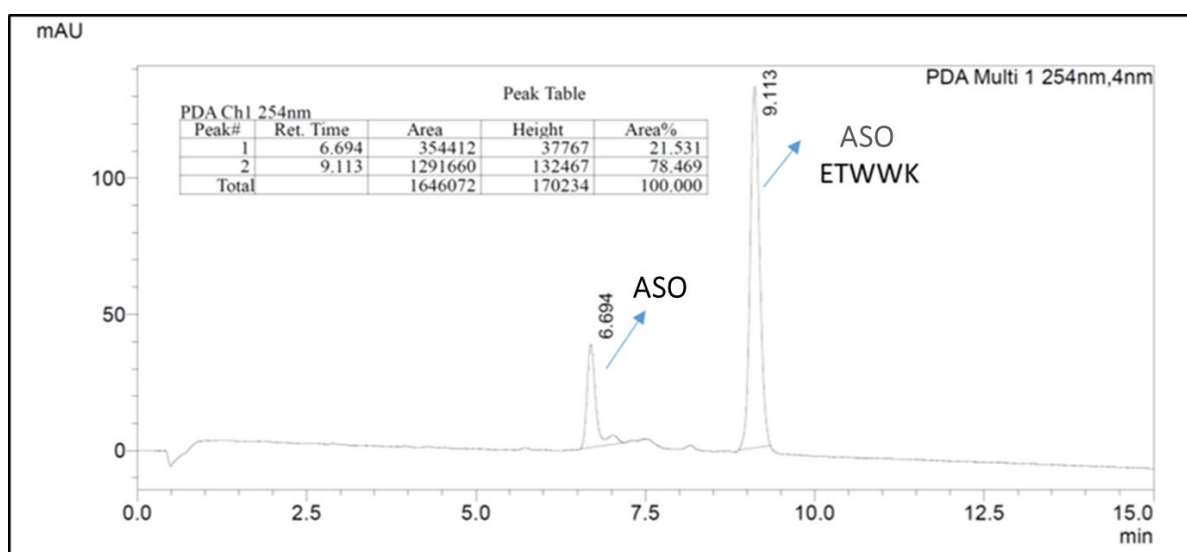


Figure 3.32: HPLC percentage conversion of ASO and ASO-ETWWK with TBTA ligand

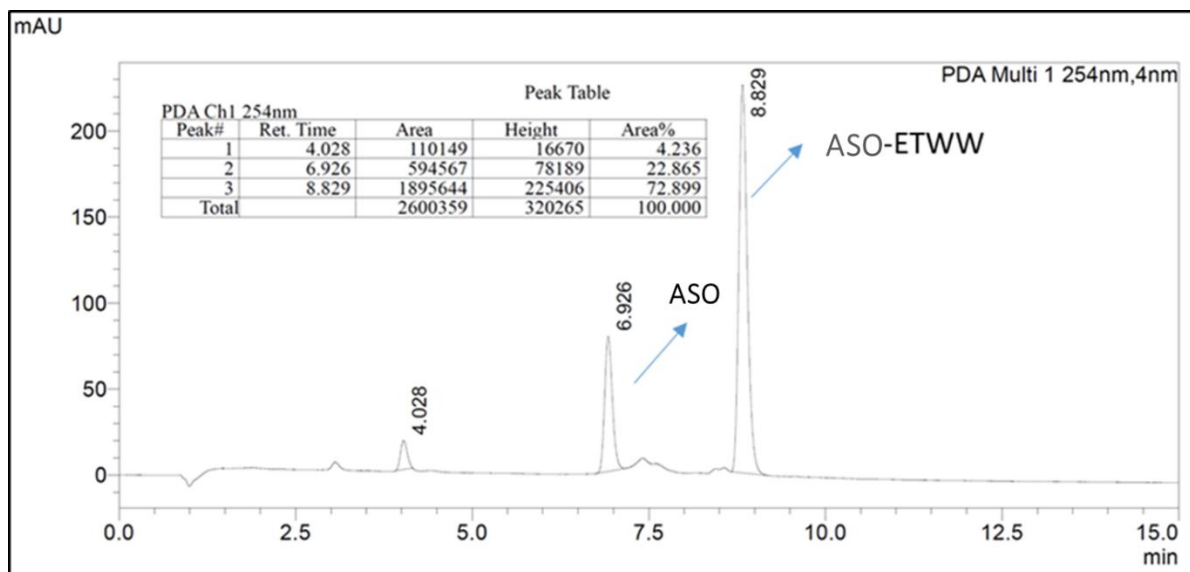


Figure 3.33: HPLC percentage conversion of ASO and ASO-ETWW with TBTA ligand.

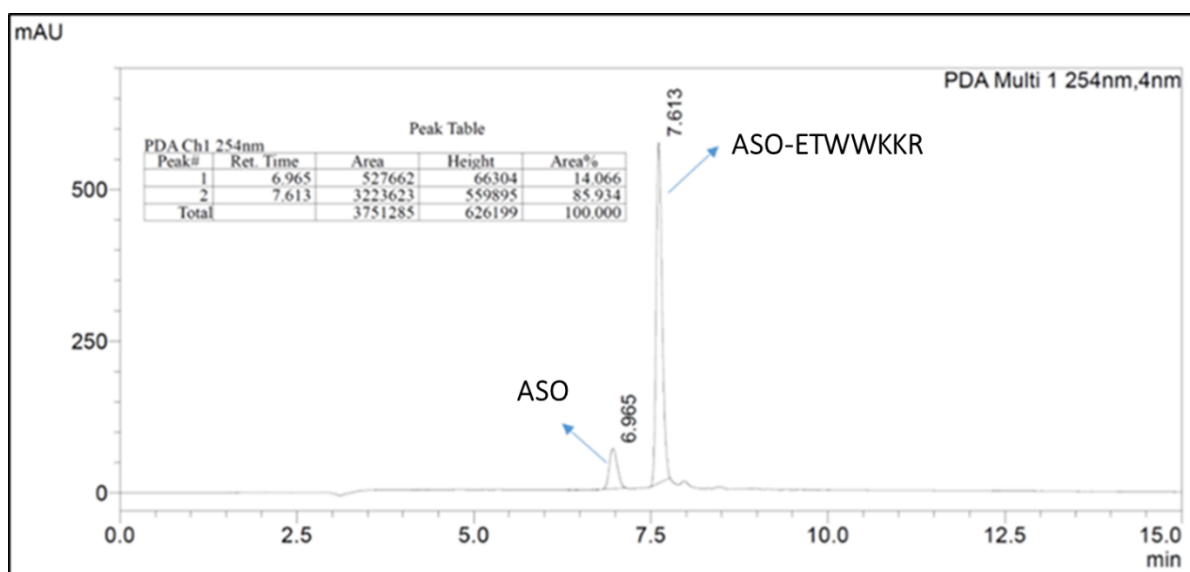


Figure 3.34: HPLC percentage conversion of ASO and ASO-ETWWKKR with TBTA ligand

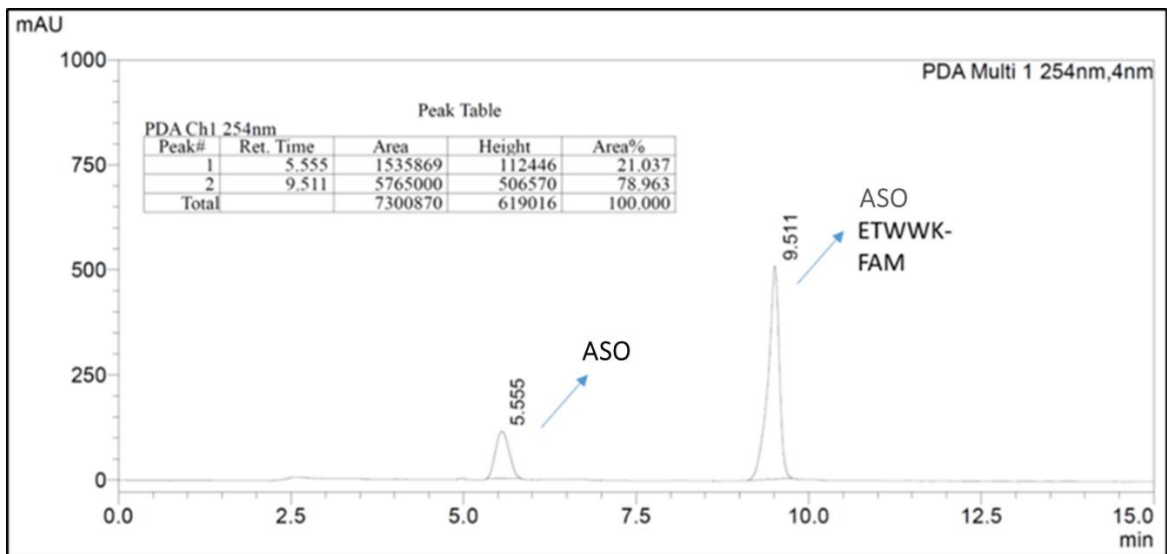


Figure 3.35: HPLC percentage conversion of ASO and ASO-FAM-ETWWK with TBTA ligand

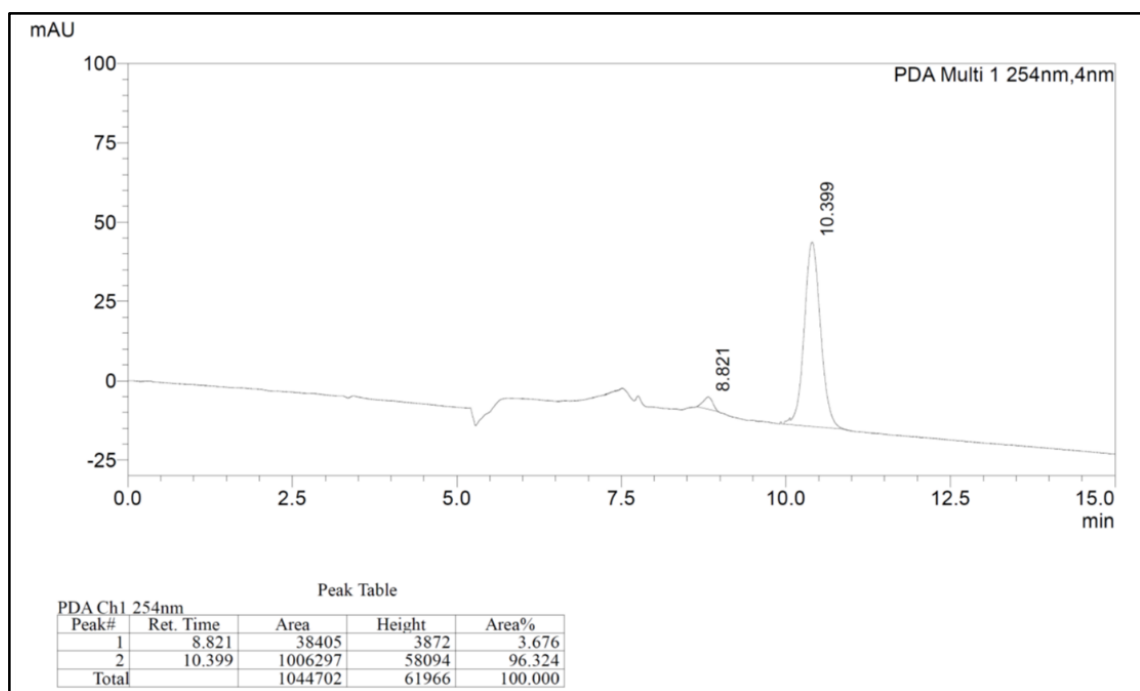


Figure 3.36: HPLC percentage purity of the ASO- ETWWK after purification

3.3.4 Monitoring of click chemistry reaction via Infrared Spectroscopy

The conversion of azide from the CPP fragment and alkyne from the ASO fragment was also monitored using IR spectra, where the corresponding azide peak at 2016.79 cm^{-1} and alkyne peak at 2043.54 cm^{-1} was not observed in the CPP-ASO conjugate product (Figure 3.37 A).

3.3.5 Mechanistic Pathway associated with uptake of Cell Penetrating Peptide ETWWK

There are majorly two mechanisms by which CPP internalize inside cell, firstly via direct translocation through the cellular membrane, which is an energy independent process, and secondly endocytosis, an energy-dependent process. Hereby flow cytometry tool was utilized to analyze the kinetics of uptake, for that purpose, two groups of cultured C2C12 cells were treated with 5-carboxyfluorescein-conjugated ETWWK and incubated separately at 37°C and 4°C for 4 hours. Flow cytometry analysis exhibits that the uptake of Fam-ETWWK by C2C12 cells was greater at 37°C compared to 4°C . These findings align with the role of endocytosis process in the cellular internalization of ETWWK (Figure 3.37 B).^{56-57, 26} Next to establish mechanistic pathway behind nuclear localization ability, our prior work with ETWW peptide clearly claimed that the presence of two consecutive tryptophan motif in the peptide sequence specifically interact with dsDNA at the major groove of DNA. Whereas, non-consecutive trp-trp units are unable to bind with dsDNA. In our present study we added single lysine residue to enhance endosomal escape of the CPP by keeping consecutive trp-trp sequence conserved.

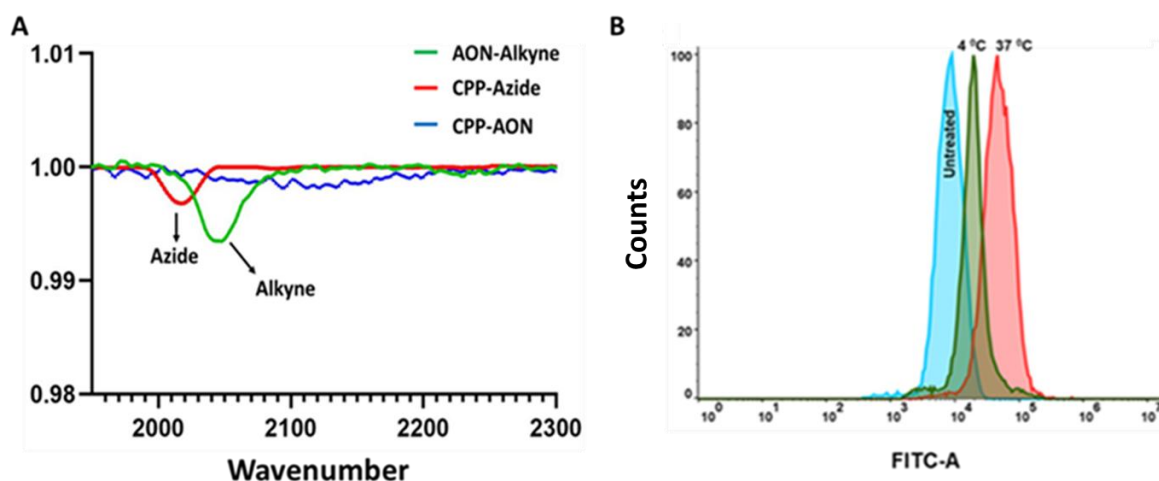


Figure 3.37: A) The progression of the reaction was tracked by monitoring the conversion of the azide and alkyne motifs into triazole moiety using IR spectroscopy. B) A cellular uptake analysis of $5\text{ }\mu\text{M}$ F-ETWWK suggests that the uptake occurs through an endocytosis pathway.

Thus, presence of dual consecutive trp-trp units in our newly designed 5-mer CPP 'ETWWK' suggests its localization within the nucleus, due to its interaction with dsDNA through a comparable mechanistic pathway.²⁶

3.3.6 Cytotoxicity of the designed Cell penetrating peptides

Based on the cytotoxicity profile of the CPPs it was observed that the addition of more than two positive charge amino acids on the CPP sequence compromise with the toxicity of the peptides. Thus except ETWWKKR, CPPs exhibits negligible toxicity even at higher concentration. This toxicity data is in alignment with the understanding that excessive positive charge residues in the CPP sequence improves cellular internalization but at the same time increase the toxicity profile of the peptides.⁵⁸ From the toxicity profile and based on the fact that presence of more than two positive charged residues hampers the coupling of CPP with negatively charged ASO, as it likely forms aggregates,²⁵ thus we eliminate CPP-4 (ETWWKKR) as potential lead candidate. However, we couple CPP-4 with ASO to evaluate the potential of our coupling strategy in presence of highly positive charged CPP sequence. Whereas, CPP-3 (ETWWR) shows cytocompatibility up to 50-100 μ M, but shows toxicity at higher doses of 200 μ M, so ETWWR was also eliminated from the lead candidature. (Figure 3.38).

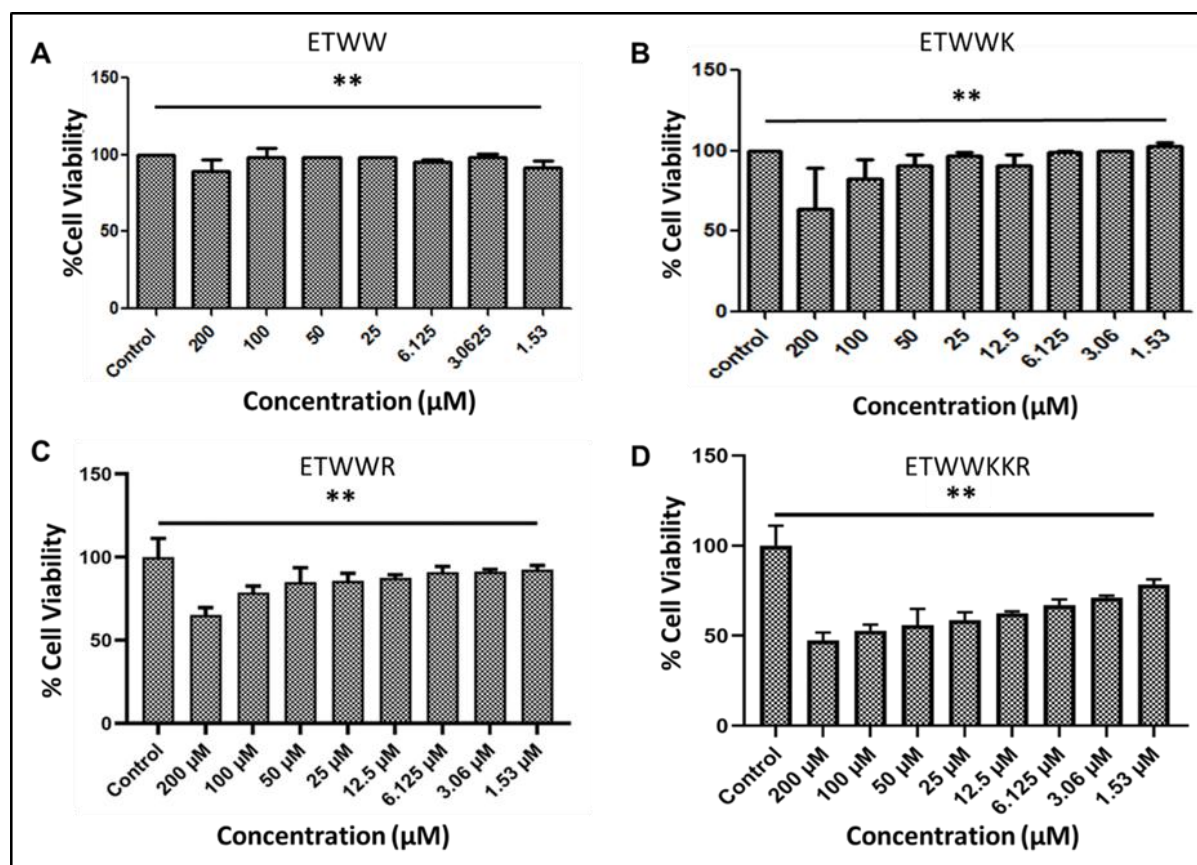


Figure 3.38: Cell survival percentage after treatment of C2C12 myoblast cells with the four CPP candidates (ETWW, ETWWK, ETWWR and ETWWKKR) identified in an increasing gradient of doses (1.53-200 μ M) for 24 h, n=3. In all data sets, significance was calculated using one-way ANOVA, **p<0.01.

To further assess the cytotoxicity of our designed ETWWK-ASO conjugate in clinically relevant cell lines, we selected the human DMD patient-derived myoblast cell line (KM1328) and human DMD patient-derived skin fibroblast cell line. In both cell line we observe that our CPP-ASO construct is non-cytotoxic even at higher doses upto 100 μ M. (Figure 3.39).

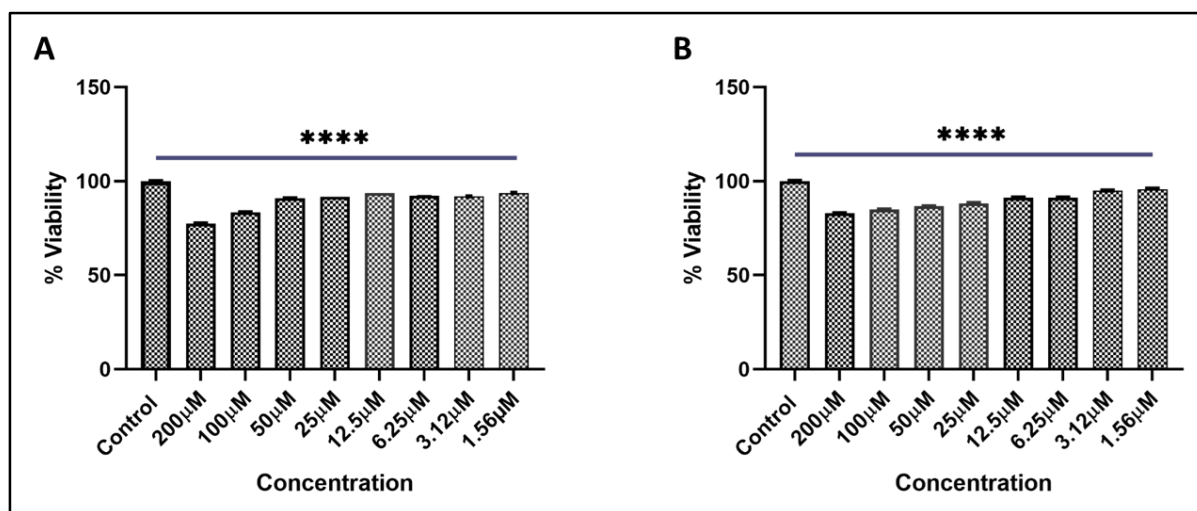


Figure 3.39: Cell survival percentage. A) After treatment of human DMD patient derived myoblast cells (KM1328) with ETWWK-ASO construct. B) After treatment of human DMD patient derived skin fibroblast cells with ETWWK-ASO construct. In an increasing gradient of doses (3.12-100 μ M) for 24 h, n=3. In all data sets, significance was calculated using one-way ANOVA, ****p $\leq$ 0.0001.

3.3.7 Cellular uptake study by immunocytochemistry and flow cytometry

The most commonly used method for measuring cellular uptake is tracking fluorescence of dye-labeled oligonucleotides based therapeutics. Flow cytometry and fluorescence microscopy are still two of the most widely used methods to measure total cellular uptake of RNA therapeutics.⁵⁹

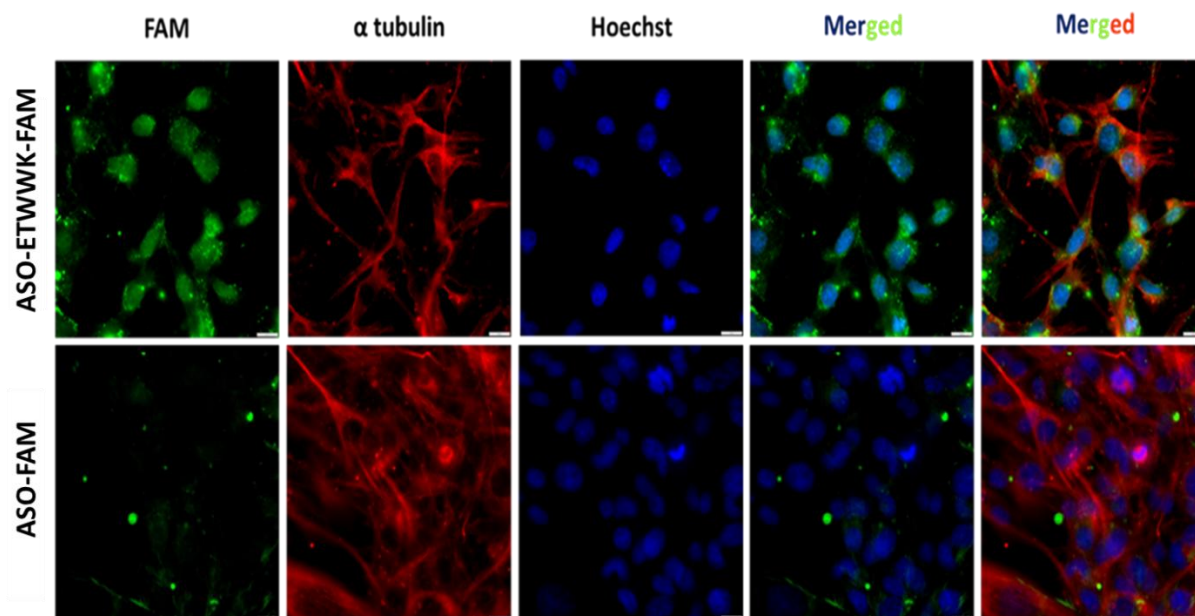


Figure 3.40: Comparative cellular uptake study between FAM conjugated ASO and FAM conjugated ETWWK tagged ASO via fluorescence microscopy upon treatment with C2C12 cells for 4 hours. Scale bar corresponds to 20 μ m.

In the current study, we chemically linked a short 5-mer CPP to a corresponding DMD exon 51-skipping antisense oligonucleotide and tested the delivery efficacy of the resulting peptide conjugated compound. The cellular internalization of the ASO and lead CPP ETWWK conjugated ASO was observed by microscopy using FAM labelled ASO and CPP conjugated ASO labelled with FAM, further nuclei staining were done with Hoechst 33258 and actin filaments with phalloidin (green color shows FAM label). Next the test compounds ASO and peptide conjugated ASO in 5 μ M concentration were incubated for 4 h in C2C12 myoblast cell lines to investigate their cellular internalization.⁵⁹ Cellular imaging clearly reveals that ETWWK conjugated ASO significantly results in efficient cellular internalization with precise nuclear localization in comparison with unconjugated FAM labelled ASO (Figure 3.40). To further demonstrate the clinical applicability in DMD patients, immunocytochemistry was performed using the DMD patient-derived myoblast cell line (Figure 3.41) to show comparative cellular uptake between FAM tagged ETWWK-ASO construct and FAM tagged ASO. In both the cell line this excellent cellular internalization ability of peptide conjugated ASO significantly facilitates the targeted delivery of the cargo ASO to its intended intracellular site of action.¹⁵

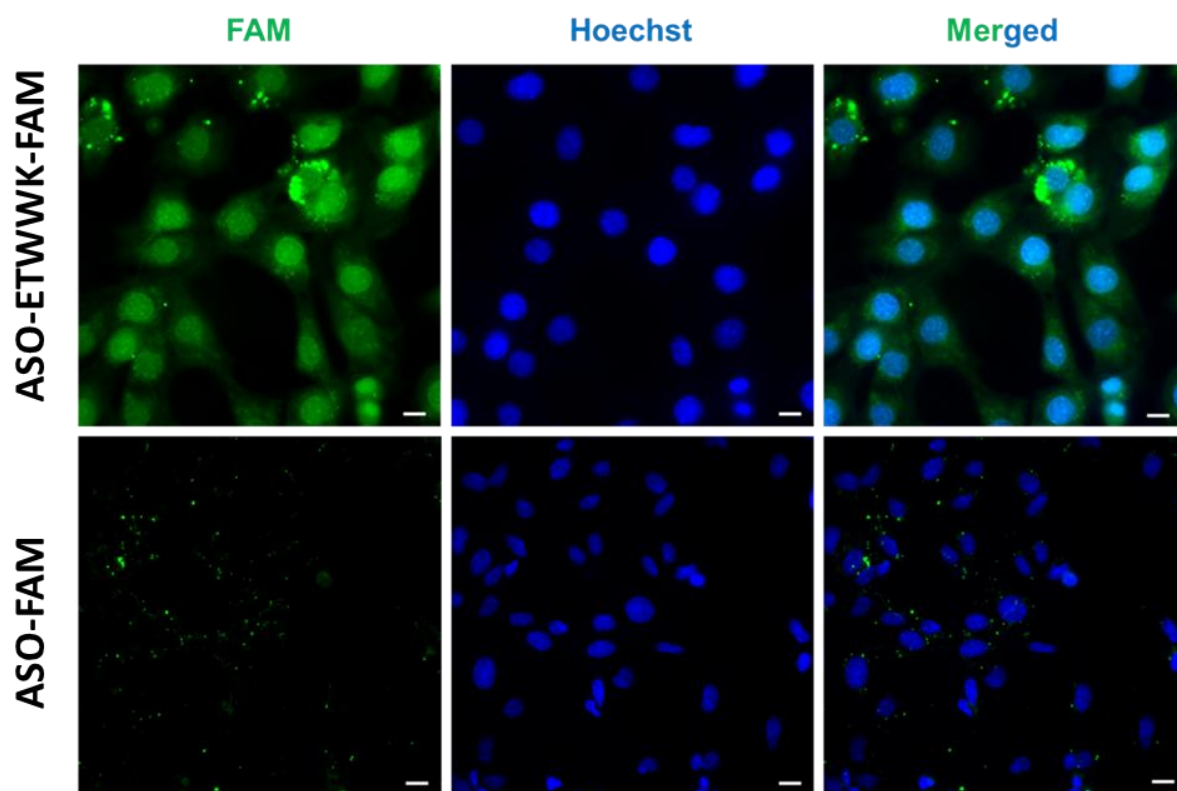


Figure 3.41: Comparative cellular uptake study of FAM tagged ETWWK-ASO construct (Upper panel) and FAM tagged ASO (lower panel) upon treatment with human DMD patient derived myoblast cells (KM1328) for 4 hours. Scale bar corresponds to 50 μ m.

Cellular uptake was further quantified by flow cytometry in human DMD patient derived cell line (KM1328) which provides more clinically relevant platform for in-vitro studies. Oligonucleotide concentration of 5 μ M, and an incubation time of 4 h were determined for testing of ASO and peptide conjugated ASO activity.

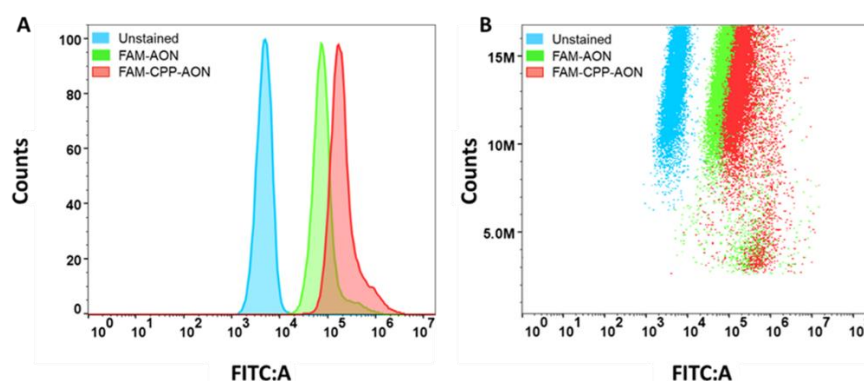


Figure 3.42: Comparative cellular uptake study between unstained (Blue), FAM conjugated ASO (Green) and FAM conjugated ETWWK tagged ASO (Red) via flow cytometry upon treatment with DMD patient derived diseased muscle cells (KM1328) for 4 hours. A&B) Comparative histogram and dot blot representations.

Flow cytometry analysis express significant cellular uptake for peptide conjugated ASO then in comparison to unconjugated FAM labelled ASO. While uptake of naked FAM labelled oligonucleotides by cells in comparison to unstained cells without the use of a delivery agent is taking place by the process known as gymnosis. But overall gymnotic delivery process leads to poor cellular uptake.^{60, 61} Whereas, in comparison to untagged oligonucleotides ETWWK conjugated ASO shows significant cellular internalization in human DMD patient derived cell line, which demonstrates enhanced ASO delivery (Figure 3.42 A&B). This advocates the potential of our designed ETWWK conjugated ASO as a promising therapeutic approach for treating individuals with DMD.

3.4. Materials and Methods

3.4.1 Materials

Chemistry

All starting materials, reagents, and solvents were purchased from commercial suppliers and used without further purification. Antisense oligonucleotides on solid support were procured from DART Bangalore. The compounds underwent routine analysis through thin-layer chromatography (TLC) performed on precoated TLC plates (Merck Silica Gel 60 F254, layer 0.20 mm) and then visualized under a UV lamp ($\lambda = 254$ and 365 nm). Solutions obtained after reactions and extractions were concentrated using a rotary evaporator. Organic solutions were dried using anhydrous sodium sulfate. ¹HNMR and ¹³CNMR spectra were acquired using a Bruker Advance 500 MHz spectrometer at a temperature of 300 K, with TMS serving as the internal standard. Mass spectra were recorded utilizing an Agilent Technologies electrospray ionization ESI; Q-TOF mass spectrometer instrument (G6530C). Infrared spectra were acquired by using Bruker FT-IR spectrometer. For high-performance liquid chromatography (HPLC) analysis, a Shimadzu C18 column (150 mm $\times$ 4.6 mm $\times$ 5 μ m) was employed.

Biology

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (M2128, Sigma), Triton X-100 (BB031, Himedia), Bovin Serum Albumin (BSA) (83803, SRL), Antimycotic antibiotic solution (Himedia A002), Fetal Bovine Serum (Invitrogen 2023-01-30), DMEM, high glucose, Penicillin Streptomycin solution (Invitrogen 15140-122), Trypsin EDTA Solution (Sigma T4174), Formaldehyde solution (Sigma-Aldrich 47608), Anti α -tubulin antibody (ab18251, Abcam, 1:1000), Hoechst 33258 (ab228550, Abcam), Alexa fluor 594 (ab150116, Abcam, 1:500), PureLink RNA Mini Kit (Cat. No. 12183018A, Invitrogen), iScript cDNA Synthesis Kit (Cat. No. #1708890, Bio-Rad).

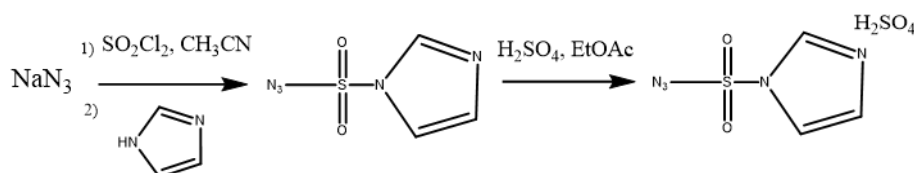
3.4.2. Methods

Synthesis of designed CPPs were performed via CEM-LIBERTY manual solid phase peptide synthesizer with rink amide resin as solid support. Next, to conjugate the synthesized CPPs to 2'-O-methyl phosphorothioate ASO via click chemistry CPPs were functionalized to N-terminal azide from N-terminal amine in solid phase utilizing synthesized azo transfer reagent. This technique of conversion of N-terminal amine to azide is simple, efficient and also cost effective. Simultaneously, the synthesized azo transfer reagent were specifically transformed into its hydrogen sulfate salt for improved shelf life, increased stability, and enhanced safety.⁶² The steps involving azo transfer and attachment of 5-carboxy fluorescein to the synthesized CPP were performed outside peptide synthesizer, by using a screw capped glass peptide vessel.

3.4.2.1 Synthesis of Cell Penetrating Peptides

The Cell penetrating peptides were synthesized employing microwave supported solid phase peptide synthesis technique utilizing CEM-LIBERTY manual peptide synthesizer as per established protocol.⁶³ Fmoc protected Rink Amide resin was taken as the solid support. A solution containing 20%v/v piperidine in DMF was used as deprotection step to remove Fmoc-protecting group of resin. After deprotection step, DIPEA (10 eq) and HBTU (5 eq) and Fmoc-protected amino acid (5 eq) were taken in DMF and stirred in vortex to make a homogeneous mixture and added in resin to allow it to couple for first amino acid. Then sequentially the deprotection and protection step were performed simultaneously until the last amino acid was attached to the resin. After final coupling of amino acid, deprotection step were performed. For attaching the azide functionality to the CPPs, conversion of N-terminal amine to azide was performed in solid phase resin as discussed under next section.⁶³

3.4.2.2 Synthesis of Azo transfer reagent, *Imidazole-1-sulfonyl Azide Hydrogen Sulfate*



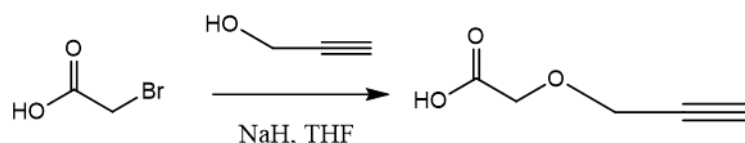
To prepare Imidazole-1-sulfonyl Azide hydrochloride, Sulfuryl chloride (0.40 mL, 5.0 mmol) was added drop-wise to a suspension of NaN_3 (0.32 g, 5.0 mmol) in MeCN (5 mL) at ice-cooled conditions, and the resulting mixture was stirred overnight at room temperature. Following this, Imidazole (0.68 g, 10 mmol) was added to the ice-cooled reaction mixture, and the resulting slurry was stirred for 3 hours at room temperature. The resultant mixture was then diluted with ethyl acetate and water. Afterward, the aqueous layer was separated and discarded, and the organic layer was subjected to successive washes with H_2O and saturated aqueous NaHCO_3 (2×15 mL). The organic layer was then dried over sodium sulfate, filtered, and the solvent removed under reduced pressure.⁶⁴ Next for the preparation of more stable and safer H_2SO_4 salt, a solution of H_2SO_4 (0.3 gm, 3.5 mmole) in ethyl acetate (2 mL) was added

drop-wise to the ice-cooled solution of imidazole-1-sulfonyl azide diluted with 10 ml of ethyl acetate with continuous stirring. The resulting mixture was chilled in an ice-bath, filtered, and the filter cake was washed with EtOAc (2 × 50 mL) to yield colorless imidazole-1-sulfonyl Azide.H₂SO₄ (0.6 gm, 57%). ⁶² ¹H NMR (500 MHz, DMSO-d₆), δ_H (DMSO-d₆, 500 MHz): 11.52 (s, 2H), 8.64 (s, 1H), 7.99 (t, J = 2.0 Hz, 1H), 7.36 (t, 1.0 Hz, 1H); δ_C (DMSO-d₆, 125 MHz): 139.5, 124.9, 124.5; LCMS calculated for diazo transfer reagent C₃H₄N₅O₂S [M+H]⁺, 174.0086, found 174.0082; LCMS calculated for diazo transfer reagent H₂SO₄ salt C₃H₅N₅NaO₆S₂ [M+Na]⁺, 293.9579, found 293.9575.

3.4.2.3 Solid phase N-terminal amine to azide transfer by utilizing imidazole-1-sulfonyl Azide. H₂SO₄

CPP attached to rink amide resin in solid phase as synthesized in section 3.3.2.1 was washed with DMF and DCM (3×5 mL). The azo transfer reactions of N-terminal amine were carried out using imidazole-1-sulfonyl azide. Hydrogen Sulfate (ISA.H₂SO₄) as synthesized. Resin attached CPP (0.1 gm) were taken in dry DMSO (3 ml), followed by addition of ISA.H₂SO₄ (3.5 eq, 94.8 mg) and DIPEA (20 eq, 0.34 ml) and allowed to shake gently in rocker for seven hours to obtain a near quantitative amine to azide conversion.⁶⁵ Now for the cleavage of CPP containing N-terminal azide from resin a cleavage cocktail containing TFA/TIS/H₂O/DTT at a weight ratio of 92.5:2.5:2.5:2.5 (w/w/w/w) was employed to prevent the reduction of azide back to amine.⁶⁶ The reaction mixture was stirred at room temperature for five hours. This choice of cleavage cocktail, and increasing the reaction time from 3 to 5 hours result in efficient conversion of amine to azide. Next, the excess amount of TFA was removed by evaporating with a continuous flow of nitrogen gas until the volume was minimized. The remaining solution was added drop wise to 40 ml of cold diethyl ether, leading to the formation of white precipitate. For further thorough precipitation, the solution in diethyl ether was kept at -20 °C overnight. The resulting precipitate was then centrifuged at 4000 rpm for 5 min, followed by dissolving the precipitate in a minimal volume of 1:1 water and acetonitrile solution, and was then subjected to lyophilization.

3.4.2.4 Synthesis of an alkyne linker for conjugation to ASO



In a round-bottomed flask, 5 mL of dry THF was taken maintaining inert atmosphere throughout the reaction. Sodium hydride (2.4 eq, 4.2 mmol, 0.16 g) was added portion wise at ice cooled condition under stirring, subsequently propargyl alcohol (1 eq, 1.78 mmol, 0.47 g) was added dropwise to the reaction mixture. Remove the ice bath and after fifteen minutes at room temperature, bromoacetic acid (1.1 eq, 1.95 mmol, 0.27 g) was slowly added portion wise. Next the reaction mixture was allowed to stir overnight at RT. After completion of starting material as monitored over TLC stained by bromocresol green, 50 mL of water was

added, and the solution was acidified with 1N HCL. Then, the aqueous layer was extracted with (25 mL× 3) ethyl acetate. The collected organic layer were dried over sodium sulfate, concentrated and used for coupling with ASO without further purification.⁶⁷ ¹H NMR (500 MHz, CDCl₃), δ_H: 4.33 (d, J= 2.5 Hz, 2H), 4.26 (s, br, 2H), 2.51 (t, J= 2.5 Hz, 1H); ¹³C NMR (125 MHz, CDCl₃): δ_C: 174.6, 78.1, 76.0, 65.6, 58.3. LCMS calculated for C₅H₅O₃ [M-H], 113.0239, found 113.0285.

3.4.2.5 Conjugation of synthesized alkyne linker to free amino linker of ASO

To functionalize the antisense oligonucleotide (ASO) with an alkyne motif for subsequent click chemistry, the free amino linker of the ASO was coupled with the synthesized short and active alkyne linker through acid amine coupling, utilizing HBTU as the coupling reagent. ASO containing free amine has been achieved by attaching the MMT-Amino C-6 CED phosphoramidite linker to the 5' end of the ASO sequence followed by deprotection of MMT group. The ASO attached to resin (10 mg) was taken in an eppendorf tube, and DMF solvent (1 mL) was added, followed by the addition of NMM base (11 µL, 10 eq). In another eppendorf the acid (SG-B-59) containing alkyne linker (5.7 mg, 5 eq) was pre-activated with HBTU (19 mg, 5 eq) in presence of the base NMM (11µL, 10eq). After fifteen minutes, activated acid was slowly added to the eppendorf tube containing resin attached to the ASO with a free amine group. The reaction mixture was then vortexed for the next 12 hours to ensure complete acid-amine conjugation. Subsequently, the resin containing ASO, now attached with the alkyne linker, was filtered off, washed with DMF and DCM, and stored for further conjugation with azide containing CPP via click chemistry.⁶⁸

3.4.2.6 Conjugation of CPPs with ASO via Click Chemistry in solid support

3.4.2.6.1 Conjugation of N-terminal Azide containing CPPs with Alkyne functionalized ASO under ligand free condition

N-terminal azide functionalized CPPs containing arginine amino acid in the sequence is conjugated efficiently with Alkyne functionalized ASO via click chemistry utilizing Copper bromide.Me₂S as a source of Copper (I) catalyst without the requirement of copper (I) stabilizing ligands. To a solid supported ASO containing alkyne linker (3 mg, 1.05 µmole, 0.00105 mmole) was taken in an eppendorf vial, followed by the addition of aza peptide (10 eq, 0.0105 mmole) dissolved in 160 µl of DMSO. Subsequently, CuBr.Me₂S (2.15 mg, 10 eq, 0.0105 mmole) dissolved in 20 µl of DMSO, and DIPEA (9.14 µL, 50 eq, 0.0525 mmole) in 20 µl of water were added to the eppendorf tube and allowed to vortex for next 3 hours at ambient temperature. Also, it is to be noted that concentration of the aza peptide in the reaction mixture should not be lower than 10 mM. Now, the resulting solid supported oligonucleotide-peptide conjugate was then filtered and washed with DMSO (5 mL×3), 50 mM EDTA disodium salt (5 mL×3, 3 times) and acetonitrile (5 mL×3) and air dried.⁷ Now, the solid supported peptide conjugated oligonucleotides were taken in a glass vial with screw cap and liquor ammonia was added to deprotect and cleave the oligonucleotide from solid support

under stirring condition at 55 °C overnight. The resulting solution was next filtered by passing through 0.2 micron syringe filter, and later washed the remaining left over resin with 70 % ethanol in water. Now, before purification of the crude product, ammonia gas was removed by continuous flow of air. The crude oligonucleotides conjugated with CPP were then purified by size exclusion chromatography using NAP-10 pre-packed with Sephadex desalting column (GE Healthcare Life Sciences) with water as eluent according to the manufacturer's protocol.⁶⁹ The fractions containing CPP conjugated ASO were then analyzed by RP-HPLC. RP-HPLC analyses were performed on a HPLC system by Shimadzu technologies employing C18 column (150 mm × 4.6 mm × 5 μm). The mobile phase consisted of a gradient from 2.5% to 70% acetonitrile with 50 mM triethyl ammonium acetate (TEAA, pH 7.0) was administered over 40 minutes at a flow rate of 1.5 mL/min, and the temperature of the column was set at 30 °C. The pressure applied ranged from 85 to 142 kgf. The detection wavelength was set at 254 nm. The purity of all conjugated CPP-ASO product isolated from NAP-10 gel purification was found to be greater than 95%, as determined by RP-HPLC analysis. The purified CPP-ASO conjugated product was then freeze dried by lyophilization for storage.

Whereas, in an alternative ligand-free condition sodium ascorbate was employed as a reducing agent to facilitate click chemistry on solid support, ensuring the copper catalyst remained in the (I) state throughout the reaction as copper (I) is prone to get oxidized to copper (II). To a solid supported ASO containing alkyne linker (3 mg, 1.05 μmole, 0.00105 mmole) was taken in an eppendorf vial and same procedure was followed as mentioned above. Now, prior to vortex additionally sodium ascorbate (11 eq, 0.0115 mmole, 2.28 mg) dissolved in 10 μL of water was slowly added to the reaction mixture. Ratio of CuBr.Me₂S: Sodium ascorbate was carefully maintained, with sodium ascorbate added in a slight excess of 1.1 eq to that of the copper catalyst. Also the addition of sodium ascorbate to the reaction mixture was meticulously performed just after the addition of copper catalyst before vortexing. Next the reaction medium was allowed to vortex for next 3 hours.⁶⁸ Now, the resulting solid supported oligonucleotide-peptide conjugate was then filtered, purified and stored following the same protocol as previously mentioned.

3.4.2.6.2 Conjugation of N-terminal Azide containing CPPs with Alkyne functionalized ASO in presence of TBTA and (BimH)₃

N-terminal azide-functionalized CPPs containing amino acids other than arginine in the sequence were not efficiently conjugated under ligand-free conditions with alkyne-functionalized ASO on solid support via click chemistry. Therefore, in this scenario, copper (I)-stabilizing ligands, such as TBTA and (BimH)₃, were employed in conjunction with CuBr.Me₂S as a source of copper (I) catalyst. To a solid supported ASO containing alkyne linker (3 mg, 1.05 μmole, 0.00105 mmole) was taken in an eppendorf vial, followed by the addition of aza peptide (10 eq, 0.0105 mmole) dissolved in 140 μL of DMSO. Subsequently, CuBr.Me₂S (2.15 mg, 10 eq, 0.0105 mmole) dissolved in 20 μL of DMSO, and DIPEA (9.14 μL, 50 eq, 0.0525 mmole) in 20 μL of water were added to the eppendorf tube. Finally, TBTA or (BimH)₃ (10 eq, 0.0105 mmole) dissolved in 20 μL of DMSO were added to the reaction mixture, and allowed to vortex for next 3 hours at ambient temperature. Also, it is to be noted that concentration of the aza peptide in the reaction mixture should not be lower than 10 mM. Now, the resulting solid supported oligonucleotide-peptide conjugate was then filtered and

washed with DMSO (5 mL×3), 50 mM EDTA disodium salt (5 mL×3, 3 times) and acetonitrile (5 mL×3) and air dried.⁶⁸ Next, the cleavage of the peptide conjugated ASO from the solid support as well as the subsequent purification and storage of the conjugated product was performed following the same procedure as discussed previously.

3.4.2.7. Synthesis of 5-carboxy fluorescein conjugated Cell Penetrating Peptide (ETWWK-FAM)

To achieve the synthesis of 5-carboxy fluorescein conjugated Cell Penetrating Peptide (ETWWK-FAM) along with N-terminal modification of peptide from amine to azide for subsequent conjugation to ASO via click chemistry, Fmoc-Lys (dde)-OH was taken. As, dde protection group on the side chain amine of lysine was selectively cleaved under nucleophilic conditions which is fully orthogonal to Fmoc deprotection conditions. This synthesis of CPP was initiated on a solid-phase Rink amide resin under a microwave-assisted peptide synthesizer, beginning with the first amino acid Fmoc-Lys(dde)-OH instead of Fmoc-Lys(Boc)-OH. The remaining coupling processes were similar to those discussed in section 3.3.2.1. Subsequent to the coupling of the last amino acid and before the Fmoc-deprotection of N-terminal of the peptide, dde group was selectively removed by using deprotection mixture outside the microwave synthesizer. The deprotection mixture was prepared by taking 1.25 gm of hydroxylamine hydrochloride and 0.918 gm of imidazole in 5 mL of NMP solvent, followed by vortex until complete dissolution. After this 1 mL of DCM was added prior to the reaction, and then resin attached peptide was gently shaken in a screw-capped glass peptide vessel with this freshly prepared deprotection mixture on a rocker for 3 hours.⁷⁰ Next, the resin was filtered off and washed with DMF and DCM (5 mL each). For FAM conjugation at side chain free amine group of lysine, 5-carboxy fluorescein was taken (5eq) in DMF (5 mL) followed by addition of DIC (10 eq) and HOBt (5 eq) and allowed to activate it for fifteen minutes. Following activation, the FAM containing acid was allowed to shake with resin attached peptide for next 72 hours under dark condition for conjugation of FAM. Next the solid support resin was washed with DMF and DCM, and it was treated with a 20% piperidine solution in DMF for N-terminal Fmoc deprotection. This N-terminal amine was then converted to azide by treating it with azo transfer reagent, following the procedure outlined in section 3.3.2.3.

3.4.2.8 Conjugation of ETWWK-FAM with ASO

ETWWK-FAM peptide containing N-terminal azide was conjugated with ASO functionalized with alkyne linker in solid support via utilizing click chemistry. This was performed in presence of additives that is copper (I) stabilizing ligand TBTA following the identical procedure as mentioned under section 3.3.2.6.2. However, in this case, the reaction was carried out under dark conditions to prevent quenching of the fluorescein fluorescence of the FAM-conjugated product.

3.4.2.9 ESI-MS analysis for the peptide conjugated ASO

Mass spectra were recorded utilizing an Agilent Technologies electrospray ionization ESI Q-TOF mass spectrometer instrument (G6530C). The masses of all ASO and CPP-ASO conjugated product mass were determined by using isocratic mobile phase (A) consisting of HFIP: TEA (400 mM: 15 mM) in water and mobile phase (B) methanol: mobile phase A (50:50), with a flow rate of 0.4 mL/min. Instrument parameters were set at an acquisition range of 400 to 3200 m/z, a scan rate were kept at 3.00 spectra/s, and ion polarity set to negative mode.

3.4.2.10 Cytotoxicity Assay

The cytotoxicity of the CPP and CPP conjugated ASO were evaluated on the C2C12 cell line using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay. C2C12 cells were seeded at a density of 5×10^5 cells per well in a 96-well culture plate and incubated for 24 hours. Subsequently, the cells were treated with various concentration of CPPs and CPP-ASO conjugated products followed by incubation at 37 °C in a 5% CO₂ environment for 24 hours. After treatment, the cells were incubated with MTT solution (5 mg/mL) in filtered PBS for 4 hours. After 4 hours, the culture medium was discarded, and the formed formazan crystals were solubilized using a solution of DMSO and methanol in a 1:1 ratio. The absorbance was measured at 550 nm using a multiplate reader.

Next to evaluate the cytocompatibility of the ETWWK-ASO construct in clinically relevant in-vitro models we checked the cytotoxicity of the conjugate product in two different human DMD patient derived cell lines: a myoblast cell line and a skin fibroblast cell line. Both cell lines were derived from DMD patients, and the cytotoxicity assessment was performed following the same procedures as described above.

3.4.2.11. Cellular imaging and uptake assay

3.4.2.11.1 Immunocytochemistry

5-Carboxy fluorescein dye (FAM) was conjugated with ASO and CPP-ASO conjugated product respectively and their respective cellular uptake and nuclear localization ability were determined via immunocytochemistry. C2C12 cells were cultured on 35 mm glass-bottom confocal dishes. C2C12 cells were then treated with FAM attached ASO and FAM tagged CPP-ASO conjugated product for 4hrs. After the treatment, cells were fixed with 4% formaldehyde solution for 30 min at 37 °C, washed thrice with PBS, and subsequently permeabilized with 0.3% Triton X-100 containing 2% BSA prepared in PBS for 30 min. After the permeabilization step the cells were washed with PBS single time after which, cytoskeleton was stained with α -tubulin primary antibody (ab52866, 1:5000, abcam) incubated overnight and anti rabbit alexa fluor 555 secondary antibody (a21429, 1:250, invitrogen) incubated for 2 hours. Then nuclear staining was conducted using Hoechst 33258 for a duration of 15 minutes. Cells were then washed again and image were taken at 40x using a fluorescence microscope (Olympus IX83).

For uptake study in human DMD patient derived cell line, cells were cultured on 35 mm glass-bottom confocal dish and follow the same protocol as described above.

3.4.2.11.2.1 Flow Cytometry Studies

Human DMD patient derived myoblast cells (KM1328) were seeded in 6-well culture plates and allowed to incubate overnight. Then the cells were treated with FAM tagged CPP-ASO and FAM attached ASO for 4 h. After treatment, the cells were washed to remove any residual culture medium and then trypsinized followed by centrifuge to pellet down the cells. The resulting cell pellet was re-suspended in PBS (0.2 mL) maintaining appropriate cell density. Then the cells were analyzed by BD LSR- FORTSEA flow cytometer to measure the intensity of green fluorescence associated with CPP-ASO and ASO respectively.

Next to investigate the endocytic mechanism of cellular internalization of Fam-tagged ETWWK, we followed a similar procedure as described above with some modifications. Human DMD patient-derived cells were seeded into two 6-well culture plates. After treatment with the dye-labeled peptide, the cells were incubated at two different temperatures, 4°C and 37°C, respectively. Following incubation, the same protocol was applied, and cellular uptake was analyzed using a BD LSR- FORTSEA flow cytometer to measure the intensity of green fluorescence associated with the ETWWK peptide at two different temperatures.

3.4.2.12 Serum stability assay for synthesized cell penetrating peptides

To evaluate the stability of the lead CPP in human plasma, a serum stability assay was performed. Initially, a stock solution of the CPP at a concentration of 10 mM in DMSO was prepared. A test compound stock at 50 μ M concentration was prepared by diluting the stock solution in acetonitrile. The frozen plasma samples were thawed at ambient temperature and then centrifuged at 14,000 revolutions per minute at 4 °C for 15 minutes. The resulting clear supernatant, approximately 90% of the total volume, was transferred to a separate tube along with 10% PBS for use in the assay. The plasma was then pre-incubated at 37 °C for 15 minutes, after which the test compound was added. Samples of 100 μ L were withdrawn from the incubated mixture at time points of 0 minutes, 30 minutes, and subsequently every 30 minutes up to 7 hours. Immediately after withdrawal, each sample was precipitated with 100 μ L of ice-cold acetonitrile.

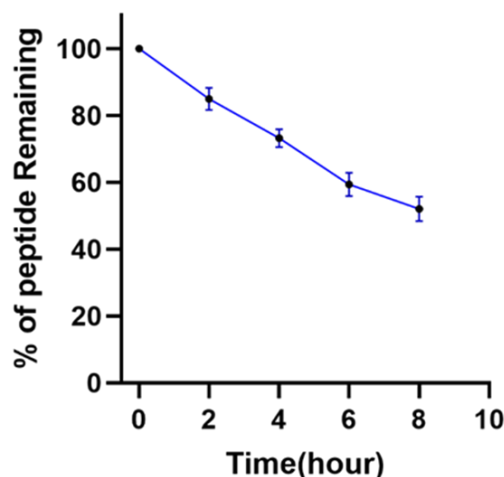


Figure 3.43: Serum stability of lead peptide ETWWK

The mixture was then vortexed for 1 minute and centrifuged at 12,000 revolutions per minute for 10 minutes. The resulting supernatant was collected and stored at -20 °C for subsequent analysis using HPLC techniques.

3.5 Conclusion

Exon skipping is a therapeutic intervention to treat DMD. The broad use of ASOs is hindered by difficulties in delivering them to target tissues, largely due to their low bioavailability. Enhancing the delivery of ASOs to their target sites, characterized by robust cellular uptake and accurate nuclear localization enhances the therapeutic potential of ASO-based treatments for DMD. Among the various delivery strategies explored, CPPs have garnered the most attention.⁷⁰ Peptide-conjugated ASOs represent a next-generation platform wherein much of the progress in improving ASO delivery and efficacy has been achieved through the conjugation of arginine-rich cationic CPPs such as (RXR)₄, B-peptide, (RXRRBR)₂, and Pips with non-anionic ASO bearing PMO backbones. However, these positively charged peptide sequences have encountered the problem of compromised toxicity at higher doses, off-target localization, and poorly tolerated in larger animals.²⁴ To address these challenges, several muscle-targeting non-cationic CPPs have been developed, such as ASSLNIA and M12. However, this muscle specific peptide shows poor cellular uptake when conjugated to PMO based ASOs and thus unable to increase the delivery or exon skipping levels in mdx mice.⁷¹⁻⁷² Some studies advocate for the use of 2OMePS backbone ASOs due to their high sequence specificity compared to PMO.¹² But the biggest challenge was associated with conjugation of cationic CPPs with anionic 2OMePS ASOs which often leads to the formation of strong aggregates.⁴⁷ Thus very few studies have explored the conjugation of negatively charged 2OMePS ASOs with CPPs specially using click chemistry.³⁴

Taken this altogether our study aims on design and development of novel CPPs focusing on the role of dual tryptophan moiety in providing excellent cellular internalization while avoiding the richness of positively charged arginine and lysine residues. This approach offers multiple benefits, including decreased toxicity at elevated doses, minimized off-target

localization, and excellent plasma stability (Figure 3.43). Moreover, our designed CPPs, owing to their neutral charge, provide a promising avenue for conjugation with negatively charged 2OMePS ASOs without the formation of aggregates, a significant drawback encountered in previous studies. In our present study the identified lead peptide, ETWWK, is a short 5-mer neutral CPP. Next, upon conjugation of ETWWK with 2OMePS ASO demonstrates significant cellular internalization with precise nuclear localization of the cargo ASO studied by flow cytometry and fluorescence microscopy in comparison to unconjugated ASO. So, herein this is the first study of conjugation of non-cationic short 5-mer CPP with excellent nuclear localization capability in delivery of negatively charged 2-OMePS backbone based oligos inside the nucleus to its site of action for achieving excellent bioavailability.

In our study apart from design and synthesis of novel short non-cationic CPPs for efficient transfer of bio macromolecule 2OMePS ASO to muscle cells, we also focus on providing an efficient, straightforward and cost effective conjugation strategy for CPP-ASO coupling. Herein, we focus on the use of click chemistry for bioconjugation as click chemistry offers superior chemoselectivity and bio-orthogonality.

Next, while performing click chemistry herein we avoided the use of expensive commercial azide and alkyne linkers commonly utilized in prior studies.^{48, 73} Instead, we focused on employing in-house synthesized short and active alkyne linkers, and on the other hand we directly converted the N-terminal amine of CPPs to azide using a lab-synthesized azo transfer reagent. This method, involving the direct transfer of CPPs N-terminal amine to azide functional motifs, reports the first instance of its application in CPP-ASO conjugation, thereby bypassing the need for costly azide linkers and thereby offering an alternative way-out. In present study we explored an area where little work is done about optimization of conjugation techniques using the 2OMePS ASO backbone, which contains a substantial PS content, through the utilization of Cu(I)-catalyzed click chemistry. In this study, the conjugation strategy employed results in significant conversion of the unconjugated ASO to peptide conjugated product. This conversion occurred both in the presence of additives such as (BimH)₃, and TBTA which promotes ligand-accelerated click chemistry, and under ligand-free conditions. However, ligand-accelerated click chemistry emerged as the optimal condition for CPP-ASO conjugation, providing higher yields and minimizing biomolecular damage. The presence of ligands not only accelerates the reaction but also significantly increases the yield. Additionally, ligands act as sacrificial ROS scavengers, protecting biomolecules from oxidative damage and reducing potential toxicity while preserving catalytic efficiency during the click reaction. Consequently, this method holds promise for synthesizing various therapeutically relevant PS-oligonucleotide conjugates, aiming to enhance their delivery capabilities.

In summary, our study represents the first short 5-mer non cationic biocompatible peptide with excellent cell penetration and nuclear localization ability. The ETWWK-ASO conjugated product effectively delivers the ASO precisely to the nucleus at its target site, where splicing occurs as confirmed by flow cytometry and fluorescence microscopy. The resulting conjugated product, coupled with the designed tailored methodology, offers a new therapeutic lead to the evolving landscape of current ASO-based therapeutic regimens most specifically to treat DMD patients by utilizing 2OMePS backbone ASO and non-cationic CPPs. These advancement holds promise to reduce the dosing frequency of current ASO-based

therapeutics. Further studies are needed to determine its potential for *in-vivo* applicability and its functional benefits in human, which will be addressed in future studies.

3.6 References

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