Abstract of Thesis

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Title	Synthesis of tetravalent PEG-conjugated antisense oligonucleotides and evaluation of their physiochemical and biological properties (４価のPEGに結合したアンチセンス核酸の合成とその物理化学的及び生物学的特性の評価)

Abstract of Thesis

This research aimed to develop an entirely new platform for the tetravalent conjugation of a 4-arm polyethylene glycol (PEG) molecule with an antisense oligonucleotide (ASO). ASOs are considered highly potential therapeutics that suffer from several limitations like short half-life *in vivo* because of either low stability towards the nuclease enzyme or their rapid renal excretion. PEGylation, where PEG is covalently attached to drug molecules, is a common technique to improve the conjugates' blood stability and retention time by reducing renal excretion and enzymatic breakdown. Thus, the pharmacokinetic behavior of the PEGylated ASO-based drugs may be enhanced by various PEG molecules, ranging from linear to highly branching or from low molecular weight to larger molecular weight. However, conjugating a 4-arm-PEG with four ASOs (4-arm-PEG-tetra ASOs) represents an entirely novel approach. As a result, I think the current study will contribute significantly to developing a new platform for future therapeutic research on ASO-based drug delivery.

The initial goal of this study was to develop a synthesis and characterization protocol to obtain pure 4-arm-PEG-tetra ASO conjugates. The choice of suitable 4-arm-PEG to conjugate with ASOs played a vital role in this regard. Here in this study, commercially available 4-arm-PEG5K-succinimidyl carboxymethyl-ester and 4-arm-PEG10K-maleimide have been selected for conjugation with ASOs with a suitable linker. I then choose an ASO that targets the *Malat1* gene (one of the most abundant gene in mammals responsible for cancer) with -NH_2 and -SH linkers, respectively, to conjugate with 4-arm-PEGs. To obtain desired 4-arm-PEG-tetra ASOs, a number of reactions in various conditions were performed between *Malat1* ASOs with amino linker or a thiol linker to conjugate with 4-arm-PEGs having an NHS ester or maleimide respectively. However, the purification and characterization procedures were the most challenging part to obtain the pure target conjugated product. Several chromatographic techniques, including reversed-phase (RP)-HPLC, ion exchange chromatography (IEX), and size exclusion chromatography (SEC), have been used for the purification of the target compound. Interestingly, RP-HPLC technique provides better yield although initially, I assumed that SEC could be a superior option due to the larger molecular weight of conjugates. The pure 4-arm-PEG-tetra ASOs were characterized by MALDI-TOF-MS measurement. As a result, the synthesis, purification, and structural analysis procedures were established, and a protocol was designed for the large-scale synthesis of 4-arm-PEG-tetra ASOs. Interestingly, the reaction yield between thiol and maleimide was found to be substantially larger (46% and 44%) than the reaction yield between amine and NHS ester (8%). The water hydrolysis of the NHS ester most likely decreased the desired yield in the second reaction. As a result, I used 4-arm-PEG-maleimide (10 kDa) conjugated with *Malat1* ASO with thiol linker for the further experimental procedure.

In the next step of my research, I performed the *in vitro* hybridizing potential of 4-arm-PEG-tetra ASOs (tetramer) with complementary RNA (cRNA) and made the comparison with parent *Malat1* ASOs (monomer). Results showed no difference in the thermal stability of duplexes formed by *Malat1* ASOs and 4-arm-PEG-tetra ASOs with cRNA. Later, the *in vitro* antisense activity of the 4-arm-PEG-tetra ASOs in NMuLi cell line using lipofectamine (transfection reagent) and gymnosis (free uptake) method were evaluated. 4-arm-PEG-tetra ASOs showed knockdown efficacy in both methods although the activity was slightly decreased compared to the equivalent parent *Malat1* ASOs. The essential point of this result was the activity of quite high molecular weight 4-arm-PEG-tetra ASOs which motivated me to investigate the gene silencing activity in different organs *in vivo*. As expected, the *in vivo* evaluation showed comparable knockdown efficacy of 4-arm-PEG-tetra ASOs with parent *Malat1* ASOs for a longer duration in few organs. This opens up new hope for future therapeutics of 4-arm-PEG-tetra ASOs.

The stability and cleavability of 4-arm-PEG-tetra ASOs were performed later in the cellular extract (CE). The key goal was to study the mechanism of the *in vitro* and *in vivo* activity of 4-arm-PEG-tetra ASOs. The result showed the conjugate was relatively stable in the CE, suggesting that 4-arm-PEG-tetra ASOs showed its activity by hybridizing with messenger RNA (mRNA) in its intact form.

Lastly, the impact of PEG conjugation on the nuclease resistance of ASOs in CE was examined. For this purpose, *Malat1* ASO sequence without any phosphorothioate (PS) was used. The stability of this sequence after PEG conjugation was observed in 90% and 50% CE, and no significant impact on PEG conjugation was observed compared to the unconjugated one. The study indicated that a comparatively high molecular weight or higher density of PEG is required to create a shielding effect of the ASOs against enzymatic degradations.

In conclusion, 4-arm-PEG-tetra ASOs consisting of 4-arm-PEG and four ASOs were successfully synthesized, purified and characterized. Later, some *in vitro* and *in vivo* biophysical properties were evaluated. In future this type of conjugating platform can be used in the conventional oligonucleotide therapeutics or duel mRNA vaccination technology.

様式７

論文審査の結果の要旨及び担当者

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論文審査の結果の要旨

核酸医薬は新たな創薬モダリティとして注目を集めており、現在世界中で難治性疾患の治療薬開発が活発に進められている。その中でもアンチセンス核酸は、疾病の原因となる遺伝子のpre-mRNAやmRNAなどと配列特異的に結合し、その機能を制御することで薬効を示す。これまでのアンチセンス核酸に関する研究の中で、生体内での安定性に優れ標的RNAに対する結合親和性を高めた各種の人工核酸が開発されてきた。こうした研究開発の進展に伴い、日米欧ではすでに９品目のアンチセンス核酸が承認されている。一方、医薬品としての応用をさらに進めるためには、まだ解決しなくてはいけない課題も残されており、アンチセンス核酸を基盤とした新たな創薬プラットフォームの開発が望まれている。このような背景のもと、申請者はアンチセンス核酸の薬物動態の改善や安全性の確保、効果の持続性の向上といった観点から、４分岐型のポリエチレングリコール（4-arm PEG）の末端に共有結合にてアンチセンス核酸を結合させた新たな核酸医薬のプラットフォームを提案し、その合成、物性評価、生物学的機能解析に取り組み、以下に示す優れた成果を得た。

１）　アンチセンス核酸と4-arm PEGとを２種類の結合様式により結合させ、その合成効率を比較検討した結果、アンチセンス核酸の末端に導入したチオール基と4-arm PEGの末端のマレイミド基との反応により、目的とする4-arm-PEG結合型アンチセンス核酸が効率的に得られることを見出した。

２）　各種の分離技術を駆使し、合成した4-arm-PEG結合型アンチセンス核酸の精製を検討した結果、逆相HPLCによる精製により目的物を高純度で得られることを確認するとともに、MALDI-TOF-MSによる構造確認法を構築した。

３）　標的RNAとの結合親和性を融解温度測定により検証し、4-arm-PEG結合型アンチセンス核酸はPEGと結合していないアンチセンス核酸と同等のRNA結合性を維持していることを見出した。

４）　細胞抽出液を処理することで、4-arm-PEG結合型アンチセンス核酸の細胞内での安定性を評価し、その高い細胞内安定性を実証した。

５）　培養細胞およびマウスに対して4-arm-PEG結合型アンチセンス核酸を投与し、十分な遺伝子発現抑制効果を確認した。また、4-arm-PEG結合型アンチセンス核酸はPEGと結合していないアンチセンス核酸とは異なる臓器特異性を明らかにするとともに、一部の臓器においては効果の持続性が向上するという知見を得た。

以上の通り、申請者は4-arm-PEG結合型アンチセンス核酸という新たな核酸医薬プラットフォームを提案し、その有用性を実験的に示すことに成功した。これらの成果は、今後の創薬科学領域の発展に大いに貢献するものであることから、本論文は、博士（薬科学）の学位論文に値するものと認める。