Mini-Review

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Drug delivery system of therapeutic oligonucleotides

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Summary **Therapeutic oligonucleotides are promising technologies. Nevertheless, improvement of their efficacy is an important issue. Introducing this drug delivery system (DDS) makes for a great enhancement for delivery of oligonucleotides to targeted tissue or cells. The strategy of DDS for therapeutic oligonucleotides is divided into four categories, A) single piece of oligonucleotide, B) oligonucleotide-ligand conjugate, C) oligonucleotide-polymer conjugate, and D) nanoparticle. In this review we will describe those basic concepts, especially for the technology of conjugating ligand. In addition, we developed a new technology, heteroduplex oligonucleotide (HDO), binding ligand-molecule to antisense oligonucleotide indirectly. We also outline α-tocopherol (a natural isomer of vitamin E) conjugated HDO.**

Keywords: Ligand conjugate, siRNA, antisense oligonucleotide, heteroduplex oligonucleotide

1. Introduction

Therapeutic oligonucleotides have rapidly progressed during the last decade and pipelines targeting a variety of disorders are now going to clinical trials (*1,2*). Despite the promising progress, improvement of efficacy *in vivo* remains a major challenge.

A variety of chemical modifications have been developed, and introducing drug delivery system (DDS) leads to greater improvement for delivery of oligonucleotides to targeted organs and cells (*3*). Especially effective delivery of small interfering RNA (siRNA) *in vivo* is difficult by itself, and needs some DDS. Strategy of therapeutic oligonucleotides is divided into four categories, as follows (Figure 1).

A) *Single piece of oligonucleotide*

Chemical modification improves stability against nuclease degradation. However, oligonucleotides are immediately egested by the kidney or accumulated in the liver and retention in blood circulation is not adequate (*4*).

Antisense oligonucleotides (ASOs) and siRNAs have a phosphorothioate (PS) backbone modification, which has two advantages. One is improvement of stability to nucleases in the body, another is improvement of binding affinity to serum proteins like albumin or others so that excretion from the kidney is delayed (*5,6*).

The first systemic ASO drug, Kynamro® targeting the liver with systemic administration was approved by U.S. Food and Drug Administration (FDA) (*7*). Targeting tissues other than the liver, however, is rather difficult and drugs for local administration have been mainly developed, for example intraocular or intrathecal administration (*8-11*).

B) *Oligonucleotide-ligand conjugate*

An approach to conjugate a ligand molecule to an oligonucleotide has been taken. Ligand conjugation improved retention of the oligonucleotide in blood and transition to the targeted organ or cell (Figure 2), as we describe later (*12*).

C) *Oligonucleotide-polymer conjugate*

Conjugating polymer to oligonucleotide can also improve retention in blood circulation. Several types of oligonucleotide can be conjugated as one particle with polymer, the size of the molecule is approximately 10 nm, compared to 5 nm for a single oligonucleotide (*3*).

D) *Nanoparticle*

There are two better-known nanoparticles, lipid

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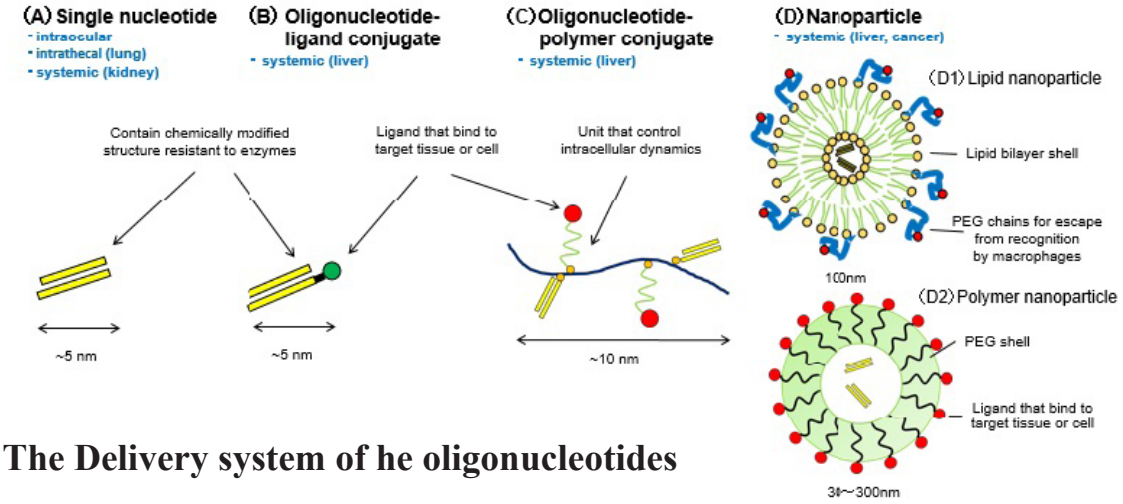

Figure 1. Delivery system of oligonucleotides. Four strategies of therapeutic oligonucleotides are illustrated. **A)** single piece of oligonucleotide, **B)** oligonucleotide-ligand conjugate, **C)** oligonucleotide-polymer conjugate, and **D)** nanoparticle. Nanoparticle has two more categories, lipid nanoparticle and polymer nanoparticle. Illustration of chemical modifications of siRNA is simplified.

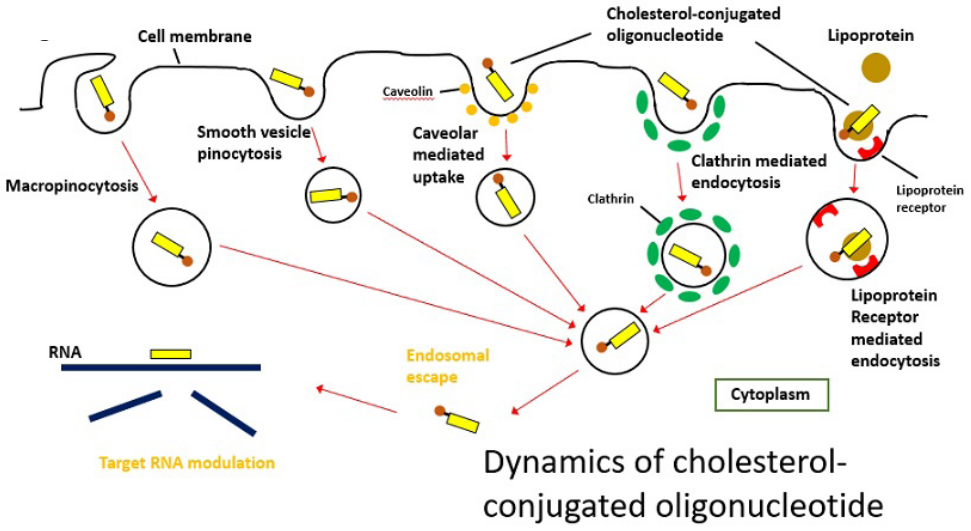

Figure 2. The intracellular and extracellular dynamics of cholesterol-conjugated oligonucleotide. Cellular uptake and intracellular trafficking of cholesterol-conjugated oligonucleotides are depicted. Cholesterol-conjugated oligonucleotide binds to lipoproteins in blood and is delivered to the liver or other tissues. Cellular uptake of the cholesterol-conjugated oligonucleotide is at least in part mediated by lipoprotein receptors. There are several endocytotic pathways other than lipoprotein receptors. Most of cholesterol-conjugated oligonucleotides are taken into early endosomes and accumulate in late endosomes and lysosomes, however, very small amount of them escape from endosome and enter into cytoplasm. Then oligonucleotides modulate the target RNA. Illustration of chemical modifications of oligonucleotides are simplified.

nanoparticle and polymer nanoparticle, in which oligonucleotides are loaded inside. Lipid nanoparticle is a small particle about a size of 100 nm, which has a shell of lipid bilayer and can load oligonucleotide in it. Polyethylene glycol (PEG) chains are able to be conjugated outside of the membrane, which help to escape recognition by macrophages. This is called a stable-nucleic-acid lipid particle (SNALP), widely used for delivery of siRNA (*13,14*). Polymer nanoparticle has a shell of PEG and oligonucleotides are loaded in it. Ligands can be conjugated on the surface. This is an aggregate of many molecules, hence the size is as large as 30 to 300 nm. Because of good retention of the nanoparticles in the blood, the development of migration to the liver and delivery to cancer tissues are now advanced (*15,16*).

In this review, we recount especially B), oligonucleotide-ligand conjugate. Conjugating ligand to siRNA has been tried since early times. Direct conjugation of ligand particle to an ASO leads to

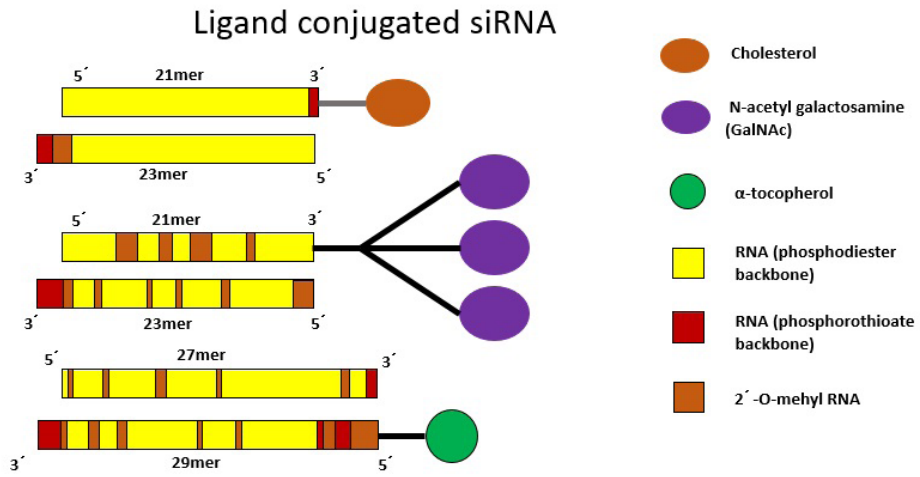

Figure 3. Schematic illustration of ligand-conjugated siRNA. Cholesterol-conjugated or N-acetyl galactosamine (GalNAc)-conjugated siRNA consists of a 21-nucleotide sense strand and a 23-nucleotide antisense strand, in which the 3' end of the antisense strand has a two-nucleotide overhang. A cholesterol or GalNAc is conjugated to the 3' end of the sense strand, respectively. Alfa-tocopherol-conjugated siRNA is a 27/29 nucleotides siRNA. The site of α-tocopherol (a natural isomer of vitamin E) conjugation is the 5' end of 29-mer antisense strand.

decrease of efficacy, and some technology to solve the problem such as using a cleavable linker is needed. Heteroduplex oligonucleotide (HDO) is a new method to indirectly bind ligand to ASO, we outline also α-tocopherol (a natural isomer of vitamin E) conjugated HDO (Toc-HDO).

2. Ligand-conjugated siRNA

siRNA is a double-stranded short RNA which can effectively downregulate a target gene that is complementary to its sequence. siRNA is recognized by Dicer and cleaved to 21/21 nucleotides double-stranded RNA in the cell. In the case of ligand-conjugated siRNA, the ligand is also cleaved by Dicer in this process so that the ligand does not negatively affect the efficacy. siRNA is then taken into an RNA induced silencing complex (RISC) *via* RISC-loading complex (RLC). The main component of RISC is argonaute (Ago) family protein (especially Ago2) that has slicer activity, RISC recognizes target messenger RNA (mRNA) that is complementary to the guide chain of the siRNA, then the target mRNA is cleaved with Ago2 (*3,17,18*). There are three examples of ligand-conjugated siRNA (Figure 3).

2.1. *Cholesterol-conjugated siRNA*

A conjugation oligonucleotide with cholesterol was one of the earliest successes in the conjugation of ligand to oligonucleotides (*19,20*). This siRNA consisted of a 21-nucleotide sense strand and a 23-nucleotide antisense strand, in which the 3' end of the antisense strand had a two-nucleotide overhang. A cholesterol was conjugated to the 3' end of the sense strand. This 21/23-mer siRNA would be cleaved by Dicer to 21/21-mer mature siRNA in the cell, which has gene silencing activity. The cholesterol conjugated siRNA targeting *apolipoprotein B* (*ApoB*) mRNA had an approximately two fold silencing effect in liver tissue compared to unconjugated using intravenous administration (*19*). In serum, cholesterol-conjugated siRNAs were found in the fraction with high density lipoprotein (HDL), low density lipoprotein (LDL) cholesterol and albumins. This meant the Cholesterol-conjugated siRNAs seemed to interact with HDL, LDL cholesterol and albumins in the serum (*12*).

2.2. *N-acetyl galactosamine-conjugated siRNA*

N-acetyl galactosamine (GalNAc) is an oligosaccharide, which has high affinity for asialoglycoprotein receptor (ASGPR). The ASGPR is a C-type lectin which is expressed abundantly on hepatocytes (500 thousand copies/cell), and its physiological function is the clearance of glycoproteins (*21*).

Triantennary GalNAc would be the best ligand for ASGPR, and the oligonucleotide is delivered much more to hepatocytes than non-parenchymal cells. The structure of siRNA was a 21/23-mer primary structure and triantennary GalNAc was conjugated at the 3' end of the sense strand. After binding of the ligand, the ASGPR-ligand complex was internalized into the cell. The ASGPR-GalNAc complex dissociated in the endosomal low pH, the receptor was recycled, while the ligand was taken into the lysosome (*5*). The GalNAc-siRNA conjugate was cleaved, then the mature 21/21-mer siRNA was discharged. The GalNAc-siRNA had approximately 5-fold potency in hepatocytes compared to the unconjugated siRNA with subcutaneous

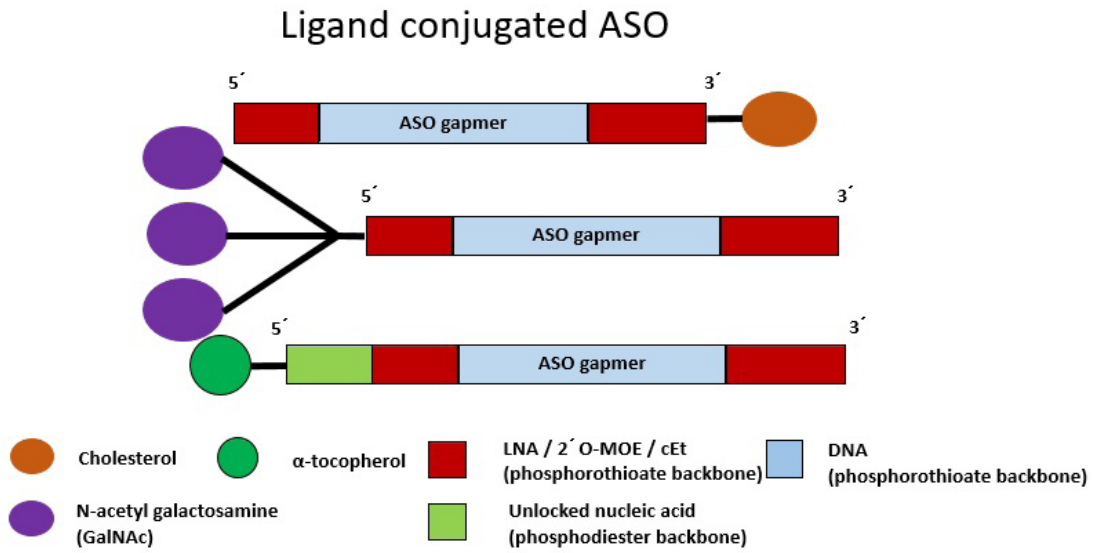

Figure 4. Schematic illustration of ligand-conjugated ASO. Cholesterol-conjugated antisense oligonucleotide (ASO) has a cleavable structure between cholesterol and ASO. GalNAc-conjugated ASO has a GalNAc ligand in its 5' end, which has a cleavable linker between the ligand and ASO. In α-tocopherol-conjugated ASO, ASO consists of 13 nucleotides. Between tocopherol and ASO are 4~7 nucleotide phosphodiester backbone unlocked nucleic acids (UNAs). LNA: locked nucleic acid, 2'-O-MOE: 2'-O-methoxyethyl RNA, cEt: constrained ethyl BNA.

administration (*22*). GalNAc-siRNA bound to plasma proteins at the rate of 94% in whole mouse plasma (*23*).

2.3. *Tocopherol-conjugated siRNA*

Ideal features of ligand molecules are essential for target organs or cells, and the target tissue cannot create the molecule. Vitamin E seemed to be most suitable for the vector because of safety and well known physiological movements (*24,25*). Hence α-tocopherol (a natural isomer of vitamin E) was conjugated to 27/29 nucleotides siRNA (Toc-siRNA). The site of α-tocopherol conjugation was the 5'end of 29-mer antisense strand. This Toc-siRNA was cleaved to the mature form 21/21-mer siRNA in the cell by Dicer. Subsequently the RISC loading and target RNA cleavage occurred. With Toc-siRNA, the gene silencing effect was much higher than the cholesterol-conjugated siRNA in liver. Only 2 mg/kg of Toc-siRNA were needed to reduce efficiently *ApoB* mRNA compared to 50-100 mg/kg of cholesterol-conjugated siRNA in the mouse liver when administered intravenously (*26*). Intestinal infusion is another way to deliver Toc-siRNA to the liver. Toc-siRNA administered as a lipid nanoparticle to the mouse large intestine in a postprandial state at a dose of 30 mg/kg reduced *ApoB* mRNA level in the liver by approximately 40% compared to the unconjugated siRNA (*27*).

3. Ligand-conjugated antisense oligonucleotide

Antisense oligonucleotide (ASO) is a single-stranded short oligonucleotide, which is chemically modified, especially 2'-O-methoxyethyl (2'-O-MOE), locked nucleic acids (LNAs) and constrained ethyl BNA (cEt). These structures improve binding affinity of the ASO to the target mRNA. Recently, gapmer ASO is predominantly used that contains two to five chemically modified nucleotides as wings at each terminal. The center of the gapmer ASO consists of a 5-10 base gap of DNA. The gapmer binds to the target mRNA and forms a DNA/RNA heteroduplex, that is recognized by RNase H and enables it to cleave the target mRNA (*5,28*). There are three examples of ligand-conjugated ASO (Figure 4).

3.1. *Cholesterol-conjugated ASO*

Cholesterol was also conjugated to ASO, not only to siRNA. Mukai *et al*. reported that a cholesterol-conjugated ASO accumulated in the liver approximately at three times a higher amount than that of unconjugated (*29*). Wada *et al*. designed a Triethylenglycol (TEG)-disulfate linker as a cleavable spacer between cholesterol and ASO. In a comparison between 5' and 3' for binding site of the cholesterol, 3'-cholesterol-conjugated ASOs accumulated more in the liver than 5'-cholesterol-conjugated ASOs. It reduced target mRNA approximately 60% in the liver with intravenous administration. Cholesterol-conjugated ASO interacted with some circulating proteins in serum, probably lipoproteins (*30*).

3.2. *GalNAc-conjugated ASO*

GalNAc was also conjugated to ASO. Until the process of endocytosis, GalNAc-conjugated ASO seemed to act similarly to the GalNAc-conjugated siRNA described above. It was suggested that the ASO escaped from endosomal compartments, and the GalNAc-ASO

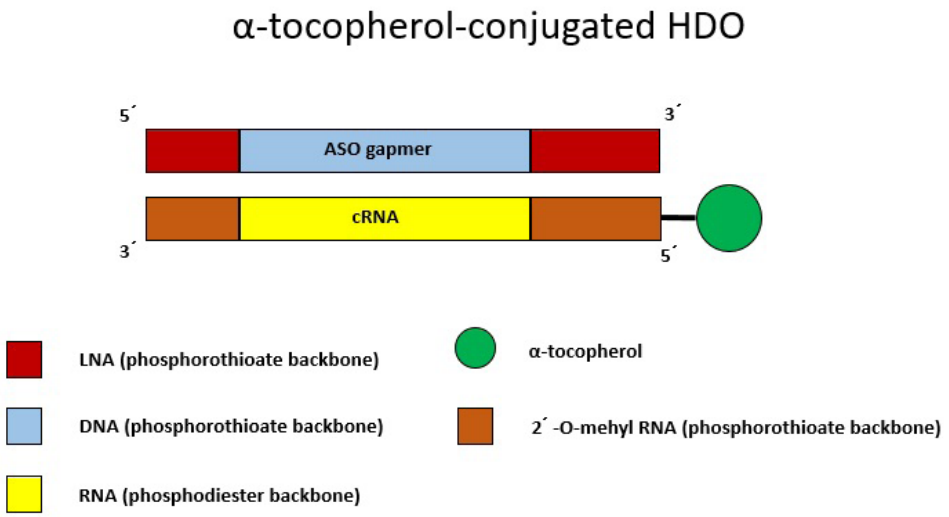

Figure 5. Ligand conjugated heteroduplex oligonucleotide. Schematic illustration of heteroduplex oligonucleotide (HDO). A HDO consists of a DNA strand gapmer which has phosphorothioate backbone and RNA strand that is complementary to the ASO (cRNA). In the cRNA strand, the nucleotides complementary to the DNA strand in the center portion are unmodified RNAs, while the nucleotides complementary to LNA in the DNA strand are phosphorothioate-modified 2'-O-methyl RNA. Alfa-tocopherol is conjugated to the 5' end of the cRNA strand. LNA: locked nucleic acid, cRNA: complementary RNA.

conjugate was cleaved at the cleavable site of the linker, then the parent ASO was discharged.

The triantennary GalNAc conjugated antisense oligonucleotide was considered to work as an hepatocyte targeting prodrug. With subcutaneous injection, it had approximately 10-fold gene silencing potency compared to the unconjugated ASO (*21,23*).

3.3. *Tocopherol-conjugated ASO*

Alpha-tocopherol (Toc)-siRNA, that was directly conjugated α-tocopherol, enhanced downregulation of endogenous genes in mouse liver compared to not conjugated. However, α-tocopherol directly conjugated ASO did not have a gene silencing effect. It was speculated that conjugation of tocopherol interfered with the gene silencing effect, then a spacer was used between ASO and tocopherol. Toc-ASO using PEG or nucleotides with phosphorothioate linkages as a spacer (second wing) also had no effect. Toc-ASO introduced nucleotides with phosphodiester linkages as second wing had reduced target gene expression.

As a length effect, Toc-13-mer (direct conjugation) and Toc-14-mer ASO had no effect, but Toc-17-mer and 20-mer ASO reduced gene expression and Toc-17-mer had an especially better effect. This was because a single nucleic acid might not be recognized by nucleases. When it was longer than a 17-mer ASO, a shorter second wing seemed to be better.

These effective Toc-ASOs were considered to reach mouse liver with full length, after that cleaved to 13-mer ASO and showed a gene silencing effect. Toc-ASO in mouse liver was more than 3.5 fold compared to tocopherol unconjugated ASO with intravenous administration. Alfa-tocopherol also improved the pharmacokinetics of the ASO (*31*).

Though Toc-ASO had a better gene silencing effect than previous ASO, its efficacy could be reduced if the "linker nucleotide" was not effectively cleaved. Hence a method to conjugate tocopherol indirectly to the ASO is desired. We discuss tocopherol-conjugated heteroduplex oligonucleotide (Toc-HDO), which we have newly created.

4. Tocopherol-conjugated HDO

HDO consists of DNA/RNA double-stranded oligonucleotide.

The DNA strand is gapmer ASO and its internucleotide linkages have phosphorothioate modifications. The RNA strand is complementary to the ASO (cRNA). In the cRNA strand, the nucleotides complementary to the LNA nucleotides of the ASO have 2'-O-methyl modifications, which are linked with phosphorothioate substitution. However, the nucleotides complementary to the DNA strand are unmodified RNAs. Alfa-tocopherol is conjugated to the 5' end of the cRNA strand (Figure 5).

The Toc-HDO targeting *ApoB* mRNA achieved great downregulation of targeted mRNA in liver tissue and also reduced serum LDL cholesterol much more than the Toc-unconjugated single strand ASO.

The Toc-HDO was accumulated approximately 5 times selectively in the liver compared to single strand ASO measured by fluorescence-label assay and quantitative real-time polymerase chain reaction (PCR) assay. However, the silencing effect of the Toc-HDO was 22.2-fold higher than the ASO at effective dose (ED) 50, we consider that the Toc-HDO was delivered much better to the hepatocytes than to the parenchymal

cells, or that the silencing effect was increased after the uptake by hepatocytes.

Serum alanine amino transferase (ALT) level was lower in the Toc-HDO injected mouse than in the ASO injected mouse with same silencing effect when targeting ApoB mRNA. Serum interferon (IFN)-γ and tumor necrosis factor (TNF)-α also did not increase.

The carrier molecule of the Toc-HDO in mouse serum was lipoprotein. Hepatocyte uptake of the Toc-HDO was at least in part mediated through the LDL receptor (*32*).

5. Conclusion

We described the outline of DDS for oligonucleotides, especially ligand-conjugated oligonucleotide. DDS is essential technology for therapeutic oligonucleotides, and further improvements are expected.

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