Review

Modification of oligonucleotides by isonucleosides incorporation and peptides conjugation

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Abstract: Synthetic oligonucleotides including antisense oligonucleotides and siRNA have shown promising therapeutic potential. However, to realize the therapeutic potential of synthetic oligonucleotides, many obstacles have to be overcome, such as their poor biological stability, non-specific activity and inadequate cell membrane permeability. In this paper, the achievements by Lihe Zhang's group in the study of isonucleotide modified oligonucleotides and oligonucleotides conjugated with cell penetrating peptides are summarized.

Keywords: Synthetic oligonucleotide; Isonucleoside; Cell penetrating peptide; siRNA

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Contents

1. Introduction

Synthetic oligonucleotides (ONs) have been investigated for decades since it was first reported that antisense oligonucleotide inhibited Rous sarcoma viral replication in 1978[1]. Acting as ribozymes and antisense strand and through RNA interference and aptamers, the uses of ONs have been expanded from gene function research[2] to therapeutic and diagnostic development[3] and other applications[4], and its target ranges from nucleic acids to proteins. However, the application of ONs is limited by the shortcomings of unmodified native oligonucleotides, such as low instability against nucleases, poor target specificity and difficulty for targeted delivery[5]. In order to improve their properties, ONs are chemically modified by a variety of approaches, such as backbone modification, sugar modification or conjugation with functional groups and molecules[6].

The essence of biological process is the specific recognitions and actions of proteins with substrate molecules. The conformations of oligonucleotides have been changed after incorporation of isonucleosides and conjugation with peptides, which may change the interactions with the corresponding enzymes. For example, the specific interactions with RNase H and RISC (RNA-induced silencing complex) have increased after incorporation of isonucleosides, which could improve the activity compared with native oligonucleotides. Moreover, peptide conjugation can significantly reduce the recognition by nucleases and significantly improve the stability of DNA and siRNA (Fig. 1). All these modifications are helpful to improve the druggability of oligonucleotides.

Various isonucleosides were synthesized and the property of oligonucleotides and siRNA modified with these isonucleosides were studied. Our previous researches on oligonucleotides modified with isonucleosides indicate that they have several advantages compared with unmodified oligonucleotides. Although the isonucleoside incorporation

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Dedicated to Professor Lihe Zhang on the occasion of his 75th birthday.

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would affect the binding affinity of the oligonucleotide with its complementary strand at different extents, the natural B form of the DNA duplexes are maintained. The oligonucleotides with isonucleosides incorporated at the 3′-ternimal show an increased resistance to exonuclease. When L-form isonucleoside was incorporated in the middle of oligonucleotides, the degradation of the complementary RNA was enhanced by reinforcing the recruitment of corresponding RNase H. The isonucleotide modified siRNAs at the 3′- and 5′-terminal showed the similar duplex thermal and serum stability as natural siRNA. And isonucleoside insertion at 5′-terminal of sense strand reduced the off target effect of unwanted siRNA sense strand, exerting silence activity.

In addition to isonucleotide modified oligonucleotides, modification of oligonucleotides with cell penetrating peptides (CPPs) is another research interest in our group. The CPPs used for oligonuclotide conjugation were designed according to computer simulation results based on empirical rules. It was found that the oligonucleotides conjugated with the designed CPPs could improve the permeability of antisense oligonucleotide and PNA through cell membrane when conjugated at 3′-end using amide bond. And CPPs were also used to conjugate with siRNA to improve the delivery of siRNA. When siRNA was conjugated with peptide at the 3′-end of sense strand, the siRNA retained their gene silencing efficiency and the off-target effect was reduced due to more difficult loading onto RISC itself. Regrettably, the membrane permeability of the conjugates was still unsatisfactory. And different processes to realize the connection between oligonucleotides and peptide were explored to provide alternative conjugation strategies for various applications.

2. Isonucleotide modified oligonucleotides

Isonucleosides represent a novel kind of nucleoside analogues in which the nucleobases are moved from C-1′ to C-2′ of the ribose, which were first synthesized in 1978[7]. Subsequently, various isonucleosides were synthesized and some isonucleosides showed potential antiviral and antitumor activity[8]. Besides, owing to the shift of *N*-glycosidic bond, the chemical and enzymatic stabilities of isonucleosides are increased.

2.1. Isonucleotide modified antisense oligonucleotides

Many isonucleotides have been synthesized in our group (Fig. 2), and the first investigated isonucleoside was L-form isonucleoside **1**, which was synthesized in 1995 through a new synthetic process that originated from D-xylose[9]. Three trimers carrying compound **1** at the 3′-terminus or in the middle were synthesized and the torsion angles in the sugar phosphate backbones were calculated using 'Amber Program' in SGI IRIS XS24[10]. It was found that L-form isonucleoside brought great steric changes in these trimers compared to regular oligonucleotides, and these alterations in torsion angles might affect the recognition of substrate by nucleases. Therefore, it was assumed that isonucleoside incorporation may improve the oligonucleotides' tolerance against nucleases and then influence the oligonucleotide's activity.

Later, the D-form isonucleoside **2** which is the enantiomer of L-form isonucleoside **1** was synthesized[11] and incorporated into oligonucleotides to compare with the oligonucleotides incorporated with isonucleoside **1**, in order to study the physiochemical property difference between the oligonucleotides incorporated with these two

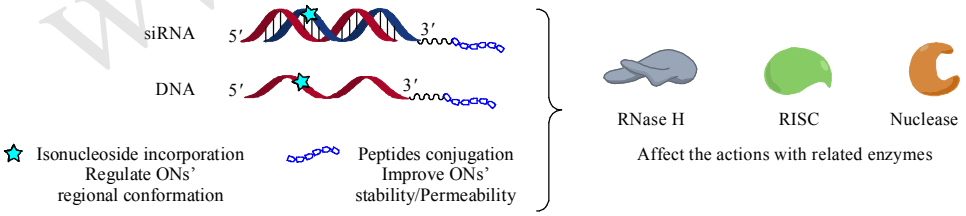


Figure 1. To improve the druggability of oligonucleotides by chemical modification, the conformations or the general properties of oligonucleotides will be changed after isonucleoside incorporation and peptides conjugation, which may affect the actions with the corresponding enzymes.

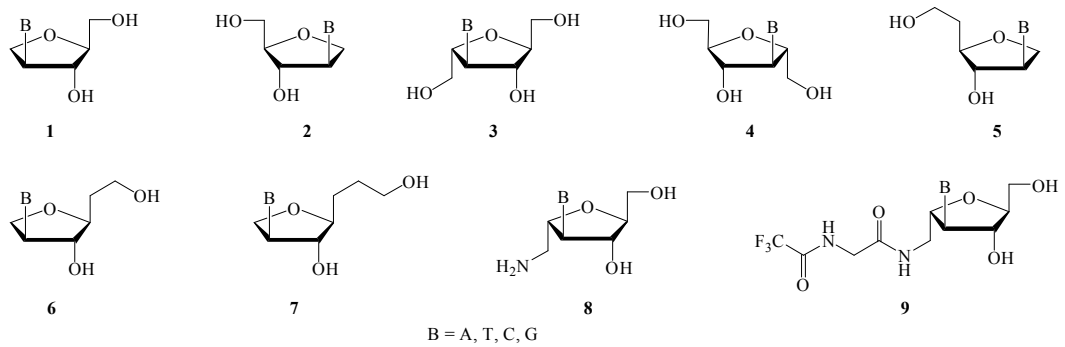
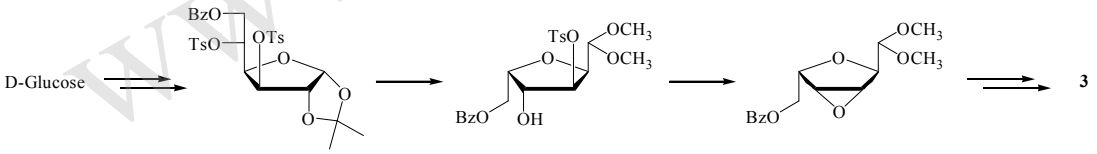


Figure 2. Isonucleosides synthesized in Lihe Zhang's group.

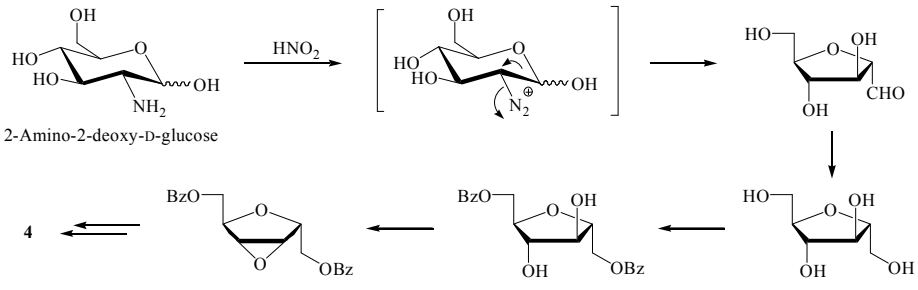
isonucleosides[12]. Meanwhile, since an RNA-like conformation of the modified oligonucleotide could increase the target RNA binding affinity and cellular uptake, isonucleoside **3** which has an additional OH group at the 1′ site of sugar ring was synthesized[13] and it was expected this additional OH group may exert beneficial influence on the hybridization properties of corresponding oligonucleotides. Oligonucleotides incorporated with the isonucleosides **1**, **2**, or **3** all showed increased stability towards snake-venom phosphodiesterase (SVPDE) while unmodified oligonucleotide was completely hydrolyzed under the same conditions[14]. This result was consistent with the result reported by Wenzel and Nair that the oligonucleotides carrying isonucleoside **2** exhibited higher resistance towards exonucleases[15], but only oligonucleotides consisted of the isonucleosides **2** and **3** retained their hybridization properties. In contrast, no typical melting was observed for a mixture of the isonucleoside **1** modified oligonucleotide with its complementary single strand. And single incorporation of either isonucleoside **2** or **3** has shown negative effect on the T_m value of the duplex, but the oligonucleotides incorporated with isonucleoside **3** give a higher T_m value than the one incorporated with isonucleoside **2**. It is well known that RNA: DNA hetero duplexes appear to be more stable thermodynamically than their DNA: DNA counterparts. Thus the reason why the oligonucleotides incorporated with isonucleoside **3** showed better hybridization ability may be because the additional hydrogen bonding caused by hydroxymethyl group of isonucleoside **3**. Besides, the hyperchromicity value for this duplex with isonucleoside **3** was much less than that of the control duplex, which may be caused by poor base stacking within the duplex.

The isonucleoside **4**, the enantiomer of isonucleoside **3**, was achieved by a simplified synthesis process[16]. In our previous work, compound **3** was obtained from D-glucose via 9 steps with a ring re-formation process leading to the formation of the sugar skeleton with the L-mannitol related configuration (Scheme 1). Similarly, the isonucleoside **4** might be prepared from L-glucose. However, L-glucose costs much more than D-glucose and it is necessary to find a practical and convenient synthetic process for the preparation of isonucleoside **4**. Finally, isonucleoside **4** was synthesized using the deamination of 2-amino-2-deoxy-D-glucose to construct the sugar skeleton in one step with the desired chirality at each asymmetric center (Scheme 2).

In the synthesis of isonucleotide modified oligonucleotides, the additional OH groups of isonucleosides **3** and **4** provide two possible phosphate backbone linkages (Fig. 3), which could be named as 1′→4′ linkage or 6′→4′ linkage, respectively[17]. Obviously, different linkage of the phosphate backbone in the oligonucleotide incorporated with isonucleoside could affect the hybridization property with its complementary strand. The oligonucleotide II (with 6′→4′ linkage) failed to hybridize with $d(A)_{14}$; however, a stable duplex of oligonucleotide I (with 1′→4′ linkage) with $d(A)_{14}$ was formed. This result indicates that the location of hydroxymethyl group may play a key role in the formation of duplex. Computer simulation showed that in I/$d(A)_{14}$, the C6 hydroxyl group of each unit is located in the groove area when hybridized to the complementary strand where it can form hydrogen bonds with water and may contribute to the stability of duplex formation, while in II/$d(A)_{14}$ most of C1 hydroxyl groups are directed to the inside of the duplex.



Scheme 1. Synthesis of isonucleoside **3**.



Scheme 2. Synthesis of isonucleoside **4**.

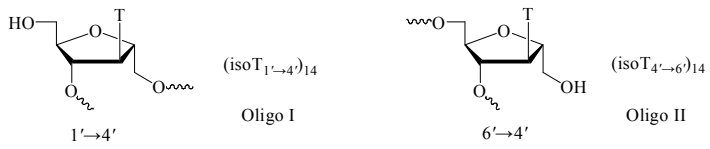


Figure 3. Linkage form of oligonucleotides modified with isonucleoside **4**.

Moreover, using the phosphotriester approach in solution phase, the octaoligoisonucleotide $(isoT)_2(isoG)_4(isoT)_2$ was synthesized from isonucleoside **4** (Fig. 4) and shown to form a parallel intermolecular G-quadruplex structure using CD (circular dichroism) spectra and capillary electrophoresis[18]. And it was shown that K^+, Na^+ and Li^+ (K^+>Na^+>Li^+) can facilitate the formation of G-quartet structures and stabilize them. Unlike the previous report in which the 6′-OH on the sugar moiety was protected by a benzoyl group, the 6′-OH was protected by a smaller allyl group during the solution phase synthesis of oligonucleotide because steric hindrance of the benzoyl group may result in lower coupling yields.

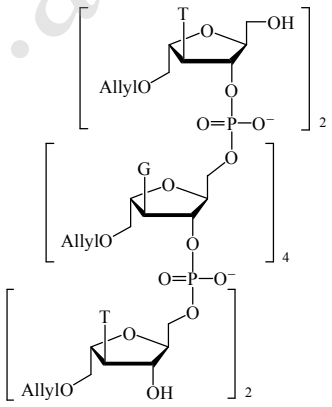
As mentioned above, the antisense oligonucleotide modified with isonucleotides could resist the hydrolysis of 3′-exonuclease and show acceptable binding ability. Meanwhile, the presence of a hydroxymethyl group on the isonucleoside could improve the ability of duplex formation. Therefore, an adenosine isonucleoside **3** was incorporated at the 3′-end or middle of the sequence 5′-AAC ATC TCC TGA GGG AAC-3′, which was complementary to spike (S) glycoprotein of the SARS-CoV (22398 22415 bp)'s encoding region mRNA[19]. Besides, the allyl group protected isonucleoside was used for studying the effect of 6′ hydroxyl group on the duplex formation of modified oligonucleotides. It was shown that the duplex with isonucleoside located at 3′-end was more stable than the corresponding mismatched sequences. However, when isonucleoside was located in the middle of the sequence, the duplex was less stable than the corresponding single-nucleoside-mismatched sequence but was more stable than multinucleoside mismatched sequence. CD spectra and computer simulation results indicated that the incorporation of isonucleoside into the oligodeoxynucleotide might only lead to the local conformational change of the duplex.

The efficiencies of RNA cleavage by RNase H digestion in the DNA/RNA duplex in the presence of different oligonucleotides were investigated. It was found that the isonucleotide modified DNA/RNA duplexes promote RNA cleavage by RNase H compared to natural DNA/RNA duplex. It is known that the efficiency of RNase H digestion is dependent on the feasibility of the formation of the DNA/RNA/RNase H complex, which was associated with many factors, such as the integrity of an A-form duplex, the width of the minor groove, and the duplex stability[20]. The isonucleotide modified oligonucleotides were more suitable for the formation of the DNA/RNA/RNase H complex which was in inverse relationship with their binding abilities to the complementary sequences. The DNA/RNA duplex formed by the isonucleotide modified oligonucleotide and its target RNA could activate RNase H. The 3′-end modified antisense oligonucleotides inhibited *S*-glycoprotein expression of SARS-CoV at the mRNA levels in insect Sf9 cells.

It was demonstrated previously that incorporation of a single isonucleoside could have a negative effect on the thermodynamical stability of the duplex. Therefore, isonucleosides **5** and **6**, as a pair of enantiomer, in which an additional methylene group is introduced at the 5-position of isonucleoside **1** and **2**, were synthesized via different synthetic procedures[21,22] to investigate whether extension of the phosphodiester linkage could improve the hybridization properties. Subsequently, a homo-oligonucleotide consisting of isonucleoside **5** was synthesized and the stability of the duplex formed with complementary single DNA or RNA strand was investigated[23]. The backbone of oligonucleotide IV (Fig. 5) was expected to be more flexible compared to the oligonucleotide containing isonucleoside **2** because of its extended phosphodiester linkage and it was expected that the distortions of the backbone in the oligonucleotide containing **2** could be compensated by the higher backbone flexibility in IV.

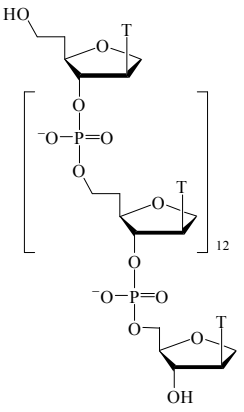
Enzymatic stability and thermal denaturation experiments showed that oligonucleotide IV has similar features as oligonucleotides made of isonucleosides **1**–**3**, such as resistance to cellular nucleases and the ability to form a stable duplex with A-DNA. However, the stable duplex of oligonucleotide IV with $d(A)_{14}$ was still formed with a slightly reduced T_m value as compared to the control duplex $d(T)_{14}/d(A)_{14}$, with no improvement compared with oligonucleotides made of isonucleoside **2**.

In the meantime, a novel isonucleoside **7** containing an extended chain with two additional methylene groups at



Oligo III

Figure 4. Structure of the octaoligoisonucleotide.



Oligo IV

Figure 5. Structure of the oligonucleotide made of isonucleosides **2** and **5**.

the 5′ position of isonucleoside **1** was synthesized to provide a more flexible phosphate-backbone to discuss the relationship between the backbone flexibility and the property alteration of modified oligonucleotides[24]. The same as oligonucleotides modified with isonucleoside **3**, when isonucleoside **7** was incorporated in the middle of oligonucleotides, the T_m value of hybrization duplexes decreased significantly compared to their native counterpart. On the contrary, the T_m values are similar as the native oligonucleotides when isonucleoside **7** was incorporated at the 5′-end or 3′-end. Oligonucleotides with isonucleoside **7** in the middle are good substrates for RNase H, in which the regional conformation change may provide a looser structure and makes the RNase H enzyme to recognize and hydrolyze the RNA strand more easily.

Considering that oligonucleotides incorporated with isonucleosides of various structures and conformations had shown negative effect on duplex stability by causing distortion in the backbone, an amino group was introduced into the isonucleoside (isonucleoside **8**) to increase the thermal stability of the isonucleoside-modified oligonucleotide with its complementary sequence[25]. The amino group may form a Zwitterionic molecule so that the amino moiety with positive charge may interact with the negatively charged phosphate backbone more strongly than the neutral hydroxyl group, thus increasing the thermal stability of the isonucleoside-modified oligonucleotide with its complementary sequence. Besides, another kind of amino-isonucleoside structure which has an elongated amino side chain (isonucleoside **9**) was also synthesized to assess the effect of chain length.

The physicochemical and biological properties of the amino-isonucleotide modified oligonucleotides were also investigated. When amino-isonucleosides were incorporated in the middle of oligonucleotides, the T_m values were decreased more obviously compared with their natural counterpart than oligonucleotides with amino-isonucleoside incorporated at the 3′- or 5′-terminal, no matter they were hybridized with DNA or RNA complementary strands. The decreased thermal stability is because the perturbation of Watson-Crick hydrogen bonding by the torsion of the backbone at the position of modification just like the previously described isonucleosides modified oligonucleotides. Especially, the elongated amino-isonucleoside (isonucleoside **9**) incorporated oligonucleotides show less effect than the normal amino-isonucleoside (isonucleoside **8**) incorporated ones when hybridized with RNA strand. The difference could be explained, as shown by computer simulation, that all isonucleoside **8** inserted oligonucleotides can form intact hydrogen bonding with complementary strand, but the hydrogen bonding was broken where isonucleoside **9** was incorporated and substituted by amide group at the position of isonucleoside **9** and that may release the torsion strain of duplex by its self-rotation. As a result, the influence on thermal stability was decreased.

In summary, oligonucleotides containing isonucleosides showed greatly increased stability towards SVPDE and acceptable affinities towards their complementary DNA or RNA. Incorporation of a single L-form isonucleoside at 3′-end of oligonucleotides have strong affinity to complementary sequences, resistance towards SVPDE, and incorporation of a single L-form isonucleoside in the middle increase the ability to activate RNase H, which is better than modifications at other positions.

2.2. Isonucleotide modified siRNA

It was demonstrated that the isonucleoside incorporation could increase the nuclease stability of the modified oligonucleotides and promote the recognition and degradation by RNase H owing to the local conformation change of hybridized duplexes. Hence, isonucleosides were also used for the modification of siRNA to improve its stability and silence potency. To date, isonucleosides **7**, **8**, **9** have been incorporated into siRNA, and their physicochemical and biological properties were evaluated.

The amino-isonucleosides **8** and **9** were incorporated into siRNA duplex to find the effect of amino-isonucleosides modification on siRNA structural stability and functional activity[25,26]. The effect of amino-isonucleosides modification on the thermal stability was similar among the duplexes between amino-isonucleosides modified oligonucleotides with complementary DNA/RNA. When single amino-isonucleoside was incorporated at 3′- or 5′-terminal of modified oligonucleotides, no obvious effect on the duplex thermal stability was observed. On the contrary, internal incorporation of one amino-isonucleoside into siRNA duplexes decreased the thermal stability. Furthermore, when two or more amino-isonucleosides were incorporated into the same strand of siRNAs duplex, T_m values decreased strikingly.

The introduction of amino-isonucleoside into siRNAs would reduce the serum stability and the degree of reduction was correlated with the number of isonucleosides incorporated into siRNAs. A single amino-isonucleoside at the sense strand exhibited a slight decrease of serum stability compared with their native siRNA. And two or more amino-isonucleosides modified siRNAs at one strand showed further weakened serum stability compared with the native siRNA.

Modified siRNAs with amino-isonucleosides **8**, **9** and isonucleoside **7** in either the sense strand or antisense strand were evaluated based on a dual-luciferase assay that renillar luciferase acts as an internal control and the fusion luciferase reporter plasmids were constructed with both target site of the siRNA duplex. It was found that no matter isonucleoside was incorporated at the terminal or in the middle of the antisense strand, the silencing potency was impaired. According to the siRNA action mechanism, the reasons for reduced silencing activity may be different between terminal incorporation and internal incorporation. When an exogenous siRNA is introduced into a mammalian cell, the 5′-end is phosphorylated by Clp1 first and then assembled to form the RISC, by which the sense strand is degraded or removed and then the antisense strand together with RISC recognizes and cleaves the target mRNA. The terminal incorporation may interfere with the Ago protein interaction with the terminal of antisense strand, thus affecting the formation of RISC complex. But the internal incorporation reduced silence activity may be related to the regional conformation around the cleavage site and consequently interfere with the RISC

complex to recognize and cleave the target mRNA. It was found that terminal amino-isonucleoside modification was more tolerable than internal modification, which implied that the step of RISC complex recognizing and cleaving the target mRNA is a more rate-limiting step for RNAi effect than RISC complex formation.

The passenger strand of siRNA modified with L-isonucleoside at 3′- or 5′-terminal could retain the silencing activities and reduce the off-target effect mediated by guide strand. The experimental data reported by us suggest that modification with the L-form isonucleosides makes the modified passenger strand more flexible, which did not influence the potency of guide strand to form the RISC, but this modification could inhibit the entrance of passenger strand and minimize the passenger specific off-target effect. It might provide an explanation for the siRNA duplex with isonucleoside substitution at the 3′-end or 5′-end of the sense strand exhibited better activity than the native siRNA. Modifications with amino-isonucleoside at the site 8 of siRNA sense strand resulted in some negative effect on siRNA activity compared with the terminal modification, which may be owing to the interference of sense strand cleavage when forming the RISC complex. Because sites 9 and 10 were the cleavage site for Ago 2 protein to cleave the sense strand, and amino-isonucleside incorporated at the site 8 of sense strand would change the regional conformation, and affect the cleavage at sites 9 and 10, thus slowing the formation of RISC complex.

3. Oligonucleotides conjugated with cell penetrating peptides

To improve the cellular uptake of oligonucleotides, CPPs were conjugated with oligonucleotides in our lab. Our group focused on the research of CPPs since 2002[27], and we found that it could improve the permeability of antisense oligonucleotide and PNA through cell membrane when it was coupled to 3′-end of antisense oligonucleotide or PNA using amide bond[27,28]. Later, CPPs were used to conjugate with siRNA to improve its delivery and several special properties were observed[29].

3.1. Introduction of CPPs

For synthetic oligonucleotides including antisense oligonucleotides and siRNA and so on to be used clinically, it requires not only high nuclease resistance and specificity, but also proper cell membrane permeability. Therefore, development of an effective delivery vector to promote permeability is essential for the development of oligonucleotides as therapeutic drugs. By comparing the two kinds of popular delivery vectors, viral and non-viral delivery systems[30], non-viral systems have prominent advantages, such as the convenience for large-scale production, the easy complex formation, and no immune system stimulation[31]. CPPs are one kind of non-viral delivery system that displays unique advantage for its lower toxicity compared to other synthetic vectors such as cationic polymers, branched dendrimers, and cationic liposomes[32]. CPPs usually refer to short positively charged peptides composed of 20 to about 50 basic amino acids residues (lysine or arginine) which can cross the cell membrane[33].

3.2. Design of cell penetrating peptides based on computer-aided modeling

From previous experience, most signal peptides that possess the ability to penetrate the cell member have a hydrophobic core consisting of a stretch of hydrophobic amino acids, basic residues near the *N*-terminal, and small neutral residues at the *C*-terminal. It implies that the hydrophobic interaction between the hydrophobic regions (h-region) composed of 5–15 amino acid residues of the peptide and the lipid bilayer of cell membrane facilitates the membrane crossing activity. Besides, the conformation of the peptide sequence also plays an important role and the ideal cell penetrating peptide sequence should have most of the h-region in the peptide sequence and easily formed α-helix with the basic residue near the *N*-terminal[34].

The CPPs used in our group were chosen from the hydrophobic pentadecapeptide corresponding to the h-region of signal sequence from Kaposi FGF[35]. And other hexa- and decapeptides peptide mimics of the pentadecapeptide were screened through the computer-aided modeling according to the empirical polarity and conformation requirement[28]. The ideal sequences should be with the lowest systemic energy in simulation of the α-helix. According to the results of simulation, two new sequences LALLA and ALLPLALLA with the lowest system energy were proposed as the cell penetrating peptide mimics for the modification of PNA and antisense oligonucleotide. And the two selected new sequences were followed by a positively charged lysine at the end of peptide to enhance the polarity and solubility, thereby enhancing membrane translocation. In conclusion, the hexapeptides mimics of LALLAK and the decapeptides mimics of ALLPLALLAK were designed to promote the membrane crossing of oligonucleotides by conjugation at the 3′-end.

3.3. Synthesis of peptide conjugated oligonucleotides

The CPPs could be conjugated with oligonucleotides through noncovalent complexation[36] with electrostatic interactions or covalent conjugation[37] such as connected by disulfide bond or phosphate diester bond and so on. Our group focused on the study of CPP's covalent conjugation owing to its wider biological applications than noncovalent complexation. There are two different strategies to form the conjugation between oligonucleotides and peptides covalently: on-line solid-phase synthesis (divergent method) and fragment conjugation (convergent) methods[38]. In the fragment conjugation, the peptide and oligonucleotide fragments synthesized individually are linked postsynthetically. In sequential solid-phase synthesis methods, the peptide and oligonucleotide fragments are assembled, sequentially on the same solid support, until the final step.

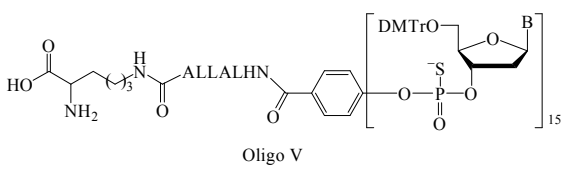
The CPP we designed is more hydrophobic according to its sequence so the method of on-line solid-phase sequential synthesis (divergent method) would be more

suitable. And the order of assembly was "peptide-first oligo-next". The peptides are assembled first by the Fmoc or Boc method, while the oligonucleotides are assembled next using the phosphoramidite method. One of the major blocks in the solid-phase sequential method is the mutual incompatibilities of the reaction conditions used for the syntheses of oligonucleotides and peptides. In addition, the protecting groups, activating and deblocking agents, used during synthesis of peptide are not more useful for assembly of oligonucleotide. Hence, proper choice of protecting groups, and other reaction conditions, is quite necessary for better yield and easy isolation of the products.

Concentrated aqueous ammonia is generally used in the cleavage and deprotection of oligonucleotides, but the phosphate diester bond derived from the aliphatichydroxyl function was unstable in concentrated aqueous ammonia. The strong basic situation of concentrated aqueous ammonia would break up the phosphate diester bond connecting peptide and oligonucleotide fragments by β-elimination[39]. It was reported that the thioester is more stable and therefore could be referred as an alternative approach to serve as the linker for the connection of peptide and oligonucleotide, but the preparation seems more complex.

Compared with the phosphate diester bond from the aliphatichydroxyl, the phosphate diester bond formed from the aromatic hydroxyl, such as the hydroxyl of tyrosine, is much more stable under the basic conditions. To alleviate the problem of unstable phosphate diester in basic situation, we have chosen linkers containing no β-protons. *p*-Hydroxybenzoic acid was designed to connect peptide and oligonucleotide fragments as linker with the improved stability. The hydroxyl modified controlled pore glass (CPG) surface was first linked to *N*-Boc, N^{α}-Fmoc protected lysine by a base-labile ester linkage, followed by the peptide synthesis by the Boc strategy. And the ester linkage between the peptide and the solid phase was not affected by the acidic deprotection procedure. Then *p*-hydroxybenzoic acid was linked to the *N*-terminus of the peptide to avoid cleavage of the phosphodiester bond by β-elimination. The resultant CPG was used for the synthesis of phosphorothio-lated oligonucleotide and finally the peptide conjugated oligonucleotides (Fig. 6) were obtained. The same group has also successfully used 2,2-dimethyl-3-hydroxypropionic acid as a linker to prepare the peptide conjugated oligonucleotide phosphorothioates[40].

Compared with solid-phase synthesis, peptides and oligonucleotides of any length were more suitable to be conjugated using the fragment conjugation in the liquid phase (LPFC) method. In LPFC method, the conjugation is completed post-synthetically after complete isolation and purification of the peptides and oligonucleotides. However, LPFC is not applicable in some cases, e.g. conjugates containing arginine rich peptides. The solution-phase conjugation could not be carried out due to precipitation of peptide, which is due to electrostatic interactions in this case. Our group overcame the peptide precipitation by the on-resin fragment conjugation method, wherein the oligonucleotide was adsorbed on an anion-exchange resin and then conjugated by passing a solution of peptide and coupling agents over the resin[40]. Alternatively, the cationic peptides were conjugated successfully to oligonucleotides under denaturing conditions.



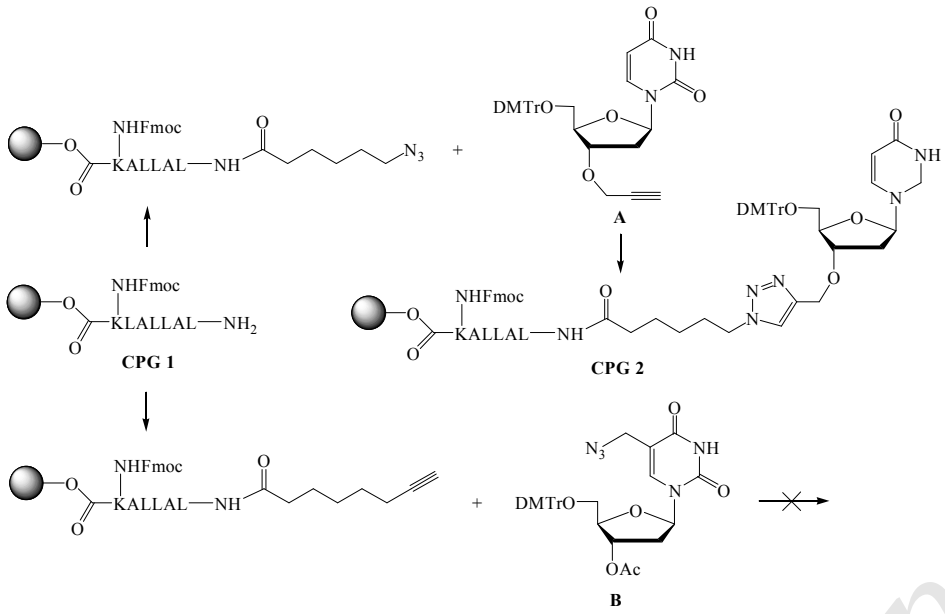
Oligo V

Figure 6. Structure of the peptide conjugated phosphorothioate oligonucleotides.

Moreover, a convenient and efficient synthetic method to covalently link the peptide with siRNA by the solid-phase click chemistry was developed in our group[29]. Following the synthetic method using phosphate diester by Chen[27], the peptide sequence was synthesized using the standard Boc strategy to an amino-ON CPG support. To expand the outreach of resin and therefore increase the collision probability between azide and alkynyl groups, a flexible C6 chain was introduced to the resin with the peptide. The terminal amide of the linker was converted into azido group or alkynyl group to react with the alkynyl group and azido group on the modified nucleoside, respectively.

The chemical modifications on the 3′-terminal overhangs of siRNA usually had less interference with the RNAi effect. In contrast, the 5′-terminal of siRNA was not so tolerated for chemical modification. Thus the 3′-overhang of siRNA was chosen as the modification site, and deoxyuridine on the 3′-overhang was attached with the 1,2,3-triazole linker and then incorporated into the oligonucleotide as part of the overhang. Both base (**B**) and sugar (**A**) building blocks were modified to allow the proceeding of click reaction with the peptide moiety. The 3′-alkynyl deoxyuridine (**A**) and the 5′-modified deoxyuridine (**B**) could both be synthesized in good yield from 2′-deoxyuridine.

According to the position of the azido group and alkynyl group, there could be two strategies to couple the modified nucleosides with the peptide derivatized CPG by click reaction. When the azido group is present on the modified nucleoside (**B**), the steric hindrance of DMT and the azide group located at the allylic position inhibit the click reaction between the azido compound and the peptide derived CPGs that have an alkynyl group. And when the terminal amino group of peptide derivative CPG (**CPG 1**) was converted to an azido group, it could react with the compound (**A**) that has an alkynyl group at the 2′-position of the sugar moiety to obtain the **CPG 2** by solid-phase click chemistry. The result showed that the load of **CPG 2** was suitable for oligonucleotide synthesis, thus the functional resin **CPG 2** was used for the synthesis of peptide–oligonucleotide conjugates on a DNA synthesizer with 1,2,3-triazole as the linker. After purification, the desired conjugates were concordant with the calculated value of the designed sequence (Scheme 3). By this means, the sense and antisense RNA oligonucleotides conjugated with peptide (LALLAK) were synthesized. For the hybridization of corresponding siRNA conjugates, naturally complementary sense and antisense RNA strands were synthesized separately.



Scheme 3. Synthesis of **CPG 2**.

3.4. Property of peptide conjugated oligonucleotides

Using the conjugation method summarized above, PNAs, oligonucleotides and siRNA are conjugated with the peptides we designed to increase their target binding and uptake by cells. To measure the membrane permeability of the oligonucleotide with peptide conjugates by Electron Spin Resonance (ESR) signal changes, stable spin label and nitroxyl free radical, 3-carboxyl-2,2,5,5-tetramethyl-pyrroline-1-oxyl as a report group, was linked to the designed conjugates.

At first, the spin-labeled PNA conjugates with the selected hydrophobic pentadecapeptide, the hexapeptides and decapeptides mimics as well as the control sample, spin-labeled PNA with no conjugation, were designed and synthesized by standard solid-phase peptide chemistry on MBHA resin using the Boc strategy[28]. According to the measurement of ESR, the three conjugates, R15T10, R10T10, R6T10, exhibited improved penetration across the erythrocyte membrane. And the peptide PNA conjugate R15T10 appears to have a faster rate of uptake than that of the hexa- and decapeptide modifiers. However, the high hydrophobicity of pentadecapeptide makes the whole PNA T10 adduct difficult to dissolve in water, which may also restrict the feasibility of its use as therapeutics. The hexa- and decapeptide modification both improve the cell membrane permeability and meanwhile show acceptable water solubility. And T_m values show that such modification has only little effect on its hybridizing behavior, which means the peptide PNA conjugates retain their binding ability to the complementary DNA. These results demonstrate the potential for peptide modification as a promising solution for improving cell membrane permeability of PNA.

Through the measurement of ESR, hexa- and decapeptide did not show obvious differences in the permeability. Considering the simple synthetic route, hexapeptide LALLAK (H-Leu-Ala-Leu-Leu-Ala-Lys-OH) was selected for oligonucleotides and siRNA modification. And because 3′-exonuclease is the major nuclease that digests native oligonucleotides in serum, to conjugate oligonucleotide with peptide at the 3′-end may improve the nuclease resistance. The antisense oligonucleotide conjugated with the hexapeptide LALLAK was synthesized, and the antisense activities targeting GLUT-1 in HepG-2 and MCF-7 cells were investigated[27]. Compared to the control sample (the conjugate of peptide-sense oligonucleotide), the oligonucleotide conjugated with the designed peptide showed up to 50% inhibition of cell proliferation in HepG-2 and MCF-7 cells, and through Northern and Western blotting analysis, the expression of mRNA and protein of GLUT-1 was shown to be decreased. It seems that the synthetic antisense oligonucleotide conjugated with hexapeptide LALLAK shows the same permeability as the expressed antisense RNA in the intact cells.

When siRNA was conjugated with the hexapeptide LALLAK by the solid-phase approach, it did not show obvious permeability which is different from antisense oligonucleotides conjugated with the same peptide mimics[29]. Through the T_m experiment, it was found that the peptide moiety may slightly reduce the siRNA duplex stability, which could be caused by the stretching of the structure of conjugate. The silence efficiency of siRNA was detected by using the siQuant^{TM} approach in HEK-293 cells. By means of the dual-luciferase assay, residual activity of mRNA was determined to assess the silencing effect. When peptide was conjugated at the 3′-end of any strand of the siRNA duplex, the silence efficiency of the modified strand will be impaired, although the degree of the reduction would be different between the antisense strand and the sense strand, and the strand without conjugation appeared to be unaffected or even enhanced in the silence efficiency. The sense strand, which shows relatively low silence efficiency, was disturbed by the peptide conjugation more obviously.

When conjugation site was at the sense strand, its silencing efficiency dropped dramatically, and when the antisense strand was modified, its silencing efficiency would improve slightly. In general, peptide conjugation would interfere with the action of the modified oligonucleotides, and may affect the competitive effect of being loaded into RISC, which are consistent with the results of isonucleoside incorporated siRNAs.

4. Conclusions

The oligonucleotides modified with isonucleosides and conjugated with cell penetrating peptides in our group have shown obvious advantages in stability, activity and membrane permeability compared with native oligonucleotides. Although there are still improvements to be made, we would anticipate that they possess the potential to become novel tools to assist the development of RNAi technology in the future.

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异核苷掺入和肽缀合修饰的寡核苷酸

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摘要: 人工合成的寡核苷酸, 包括反义寡核苷酸、干扰RNA等, 显示了优异的临床潜在应用价值。但要实现合成的寡核苷酸的广泛临床应用, 必须要克服一些障碍性问题, 如该类结构的不稳定性、非特异性作用及难以跨膜吸收等。本文综述了张礼和院士课题组在异核苷掺入及肽缀合寡核苷酸方面的研究成果。

关键词: 人工合成寡核苷酸; 异核苷; 透膜肽; 干扰RNA

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