Chem. Res. Chinese Universities, 2020, 36(2), 164—170 doi: 10.1007/s40242-019-0033-2

Review

Artificial Nucleotide-containing Aptamers Used in Tumor Therapy

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Abstract The high pharmaceutical cost and multi-drug resistance in tumor therapeutic agents hinder the further application of chemotherapy in tumor therapy. Artificial modified nucleic acid aptamers have the advantages of high binding affinity, programmability, and easy synthesis. Thus, the rational design of artificial modified aptamers is expected to provide a versatile platform for the optimization of chemotherapy agents. In this review, we summarize the modification strategies and the application of the artificial modified nucleotide-containing aptamers, aiming to provide a promising step toward aptamer-related chemotherapeutic agents.

Keywords Aptamer; Artificial nucleotide; Functional nucleic acid

1 Introduction

Currently, tumor is a major killer of human health[1]. Though various tumor therapeutic agents have been developed, some problems, such as high pharmaceutical cost and multi-drug resistance still hinder their further application[2]. Thus it's necessary to develop safer and more efficient chemotherapeutic agents[3]. Nucleic acid aptamer is a sequence of nucleic acids those can specifically bind with the target molecules[4—7]. With its high binding affinity, programmability, and easy synthesis, aptamers can effectively internalize the target cells, avoiding the interception-caused drug resistance[8,9]. Therefore, aptamers hold a great potential to overcome the bottleneck of chemotherapeutic agents[10].

Aptamer is a sequence of nucleic acids made of four kinds of natural nucleotides as building blocks. Different aptamers have various spatial structure and function[11]. Therefore, *in vitro* screening technique SELEX(systematic evolution of ligands by exponential enrichment) is widely used to screen nucleic acid sequences that can bind to specific targets[12,13]. However, for some tumor cells, it is still hard to obtain an aptamer with high binding affinity. It is mainly caused by the spatial conformation limitation of natural nucleotide consisted aptamers[14]. Introducing artificial modified nucleotides into aptamers can significantly enlarge the spatial conformation of aptamers[15], improving the success rate of SELEX process[16]. At the same time, some artificial modified nucleotides can also show anti-tumor effect. As mentioned above, aptamers with artificial modified nucleotides are highly expected to promote the tumor therapy strategies[17,18].

Recently, aptamer-related reviews usually focus on natural nucleotide-consisting aptamers and their applications and few talk about the artificial modified nucleotide-containing aptamers[19]. Therefore, we introduce the artificial modified nucleotide-containing aptamers and their application in tumor therapy. In this review, we summarize the modification strategies and new properties of the artificial modified nucleotide-containing aptamers. And we also review the applications of the modified aptamers as tumor therapeutic agents and drug-delivery systems. At the end of this review, we present the opportunities and challenges of the artificial modified nucleotide-containing aptamers used in tumor treatment, aiming to provide a promising step toward aptamer-related chemotherapeutic agents.

2 Generation of Artificial Nucleotide-containing Aptamers

Chemical modification is the process of changing molecular activity by adding or removing some functional groups from molecules[20—22]. Chemical modification can improve the stability, water solubility, biocompatibility and other properties of the target molecule to optimize the target molecule[23—25]. Chemical modifications add functional groups, such as methylation, ethylation, and the like[26,27]. In addition, the chemical modifications of nucleotides also include the insertion of artificial bases[28,29].

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Received October 31, 2019; accepted November 14, 2019.

Supported by the National Natural Science Foundation of China(Nos.21904037, 21675120) and the National Key R&D Program of China(No.2017YFA0208000).

2.1 Functionalization

The chemical function of natural nucleic acid is very limited, which restricts its application in biomedical field. The researchers found that the DNA ligase also acts as a normal ligation for the modified nucleotides. Therefore, the monomer nucleotide can be modified to give it more chemical functions, and then the monomer can be connected to nucleic acid molecules with DNA ligase for biomedical research. Hili *et al*.[30] selected the 5'-phosphorylated trinucleotide as the nucleotide monomer and successfully connected it with the T4 ligase to obtain the required nucleic acid. In addition, they found that the reaction could follow a known DNA template and regenerate the template in the reaction, creating a virtuous circle of reactions. Therefore, it is possible to synthesize functional nucleic acid aptamers by this method.

In addition to functionalizing each nucleotide in the aptamer and synthesizing the aptamer artificially, modifying the end nucleotide of the aptamer is also a promising approach. Recently, researchers found that functionalization of only a few specific nucleotides in the nucleic aptamers can also significantly improve nuclease resistance of aptamers, which greatly reduced the difficulty of chemical modification of the nucleic aptamers. In addition, nuclear aptamers, with terminal nucleotide functionalization can improve their chemical reactivity through this functionalization while improving their own nuclease resistance. Zhao *et al*.[31] used sulfhydryl group to modify the end of AS1411 aptamer, which not only improved aptamer stability, but also improved its chemical reactivity, which made it able to bond with gold nanoparticles through Au—S bond. Therefore, this method will significantly improve the chemical reactivity of aptamers, which is expected to realize the combination of functionalized aptamers and other molecules and build a more effective anti-tumor drug system.

DNA aptamers can form DNA-protein complexes by specifically binding to targeted proteins, which has many applications in the field of biomedicine. In addition, modification of the DNA aptamers can improve or give it selectivity for a particular protein. However, this labeling method needs to be carried out by biological orthogonal method in physiological environment, so as to avoid the interference of biological biochemical reaction. Recently, Wang *et al*.[32] found a chemical solution that promotes DNA-protein specific binding. Additionally, they successfully identified and bound proteins on lysine residues with F-carboxy-modified DNA aptamers specific through biological orthogonal methods. At the same time, they also demonstrated that the aptamers not only have the chemical functions of the aptamer itself, but also have the targeting function of F-carboxyl group. This also shows that chemical modification can effectively improve the reactivity of DNA aptamers, giving them advantages that natural nucleic acids do not have.

2.2 Artificial Bases

Recently, researchers have suggested that hydrophobicity is a key link in the self-assembly of large molecules, and that DNA can rely on this hydrophobicity to achieve double-stranded self-assembly. Moreover, hydrogen bonds in DNA can be formed without complementary base pairs, as long as two hydrophobic base pairs match in size and have enough π-π overlap between them. Therefore, the researchers started thinking about ways to extend the variety and applications of DNA by using synthetic bases to build synthetic DNA double strands. In order to explore the effect of this hydrophobic action on the formation of DNA double chain, Wang *et al*.[33] successfully synthesized an FNA(fluorinated nucleic acid) by using the hydrogen bonding between the artificial base F [3,5-bis(trifluoromethyl)benzene](Fig.1). In addition, they used another hydrophobic base M(3,5-dimethylbenzene) for comparison, demonstrating the role of hydrophobic action in the self-assembly process and this hydrogen bonding would not affect the existing hydrogen bonding between A-T and C-G. Due to the expansion of existing base pairing functions, this synthetic nucleic acid has great potential in biomedicine.

In general, aptamers made up of natural nucleotides tend to be easily hydrolyzed by nucleases, so it is necessary to carry

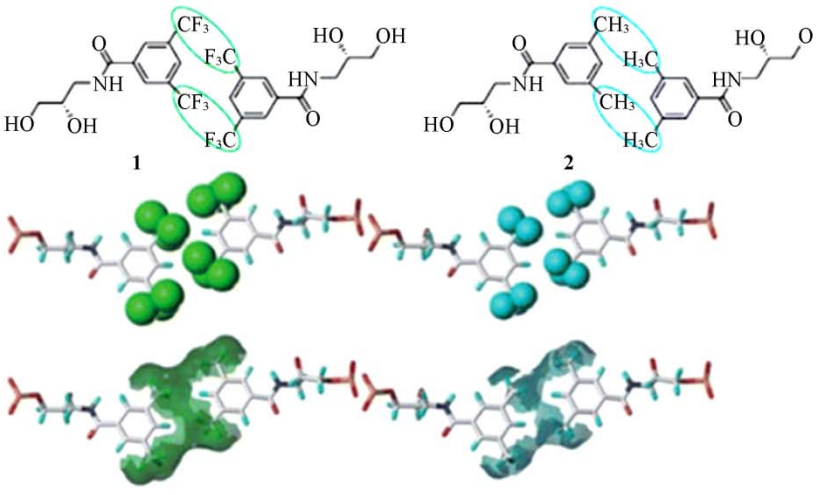

Fig.1 Structure, molecular model and the Connolly surface models diagrams of F base and M base[33]

1 and 2 are the structures of the F and M bases, respectively. The molecular models under the structures show the distance between the base and the Connolly surface models show the hydrophobic continaity of the base. Complementary pairing of artificial bases.

out effective chemical modification, such as adding artificial bases to improve the stability of aptamers. Kimoto *et al*.[34] synthesized a DNA aptamer with the addition of hydrophobic base Ds(7-(2-thienyl)imidazo[4,5-b] pyridine). Moreover, they demonstrated that hydrophobic base Ds can significantly enhance the compatibility of aptamers and effectively increase the chemical function and diversity of DNA molecules, providing a new method for improving aptamers compatibility. Recently, Matsunaga *et al*.[35] synthesized DNA aptamers containing Ds artificial bases by a similar method and confirmed the increased affinity by measuring the binding ability with the target. Besides, they identified the position of each Ds base by probe hybridization and optimized the DNA aptamers by adding small hairpin DNA to improve the stability of the DNA aptamers. This kind of DNA aptamers containing artificial bases has better targeting ability and higher stability in the treatment of diseases.

Artificial bases have been shown to disrupt the sequence of existing base pairs to create new unnatural nucleic acids. Therefore, the researchers proposed an artificial aptamer-based disease treatment program, which constructed a protein-specific aptamer by artificial nucleotides to target receptor proteins for disease treatment. Biondi *et al*.[36] constructed the first artificial aptamer that can target and specifically bind to PA63 by using artificial bases Z, P and natural bases A, T, C and G. PA63 is a protective antigen of anthrax bacillus, which can open a toxin channel on the surface of the host cell and transfer other toxin factors of anthrax bacillus into the host cell to make the host sick. This artificial base-based aptamer can target PA63, thus blocking other toxin factors from entering the host cell and achieving the treatment of diseases.

3 Tumor Therapeutic Agents Based on Artificial Nucleotide-containing Aptamers

Gene therapy regulates gene expression and function by binding to the target[37—40]. The first is to inhibit the growth and spread of tumor cells. Tumor therapeutic agents can promote the death of tumor cells by targeting selected proteins to block the spread of tumor cells[41—44]. The second is the activation of the body's immune system, to achieve tumor cell clearance and accelerate tumor cell apoptosis[45—49].

3.1 DNA Aptamers

AS1411 is a kind of DNA aptamers that can specifically bind to nucleolus protein on the surface of tumor cells. Hence it can induce apoptosis of tumor cells without affecting the physiological activities of normal cells. However, it also has the disadvantage of poor stability. Fan *et al*.[50] found that the insertion of 2'-deoxyinosine could increase the stability of the aptamer AS1411. In addition, through experiments, they confirmed that the artificially modified DNA aptamer could inhibit the proliferation of breast cancer cells by blocking DNA replication, so as to achieve the goal of tumor therapy. Therefore, chemically modified AS1411 is expected to be an effective and safe tumor therapeutic agent. Interferon-γ is an anti-tumor cytokine, but it is prone to cause its own adverse immune response, so it is necessary to develop substances that bind to interferon-γ to neutralize antibodies. Matsunaga *et al*.[51] used an artificial base(Ds-Px)-modified DNA aptamer to target interferon-γ, which inhibits the biological activity of interferon-γ and acts as a neutralizing antibody(Fig.2). In their experiments, they found that the survival rate of recombinant DNA aptamers added with tiny hairpins increased significantly, suggesting that recombinant DNA aptamers improved the compatibility and stability of the aptamer. This recombinant DNA aptamer can reduce the adverse immune response in treatment and can be used for mild tumor therapy.

KRAS gene can control the growth path of cells, and KRAS gene mutation will cause uncontrolled cell proliferation and carcinogenesis. Therefore, if the expression of KRAS gene in tumor cells can be controlled, the development process of

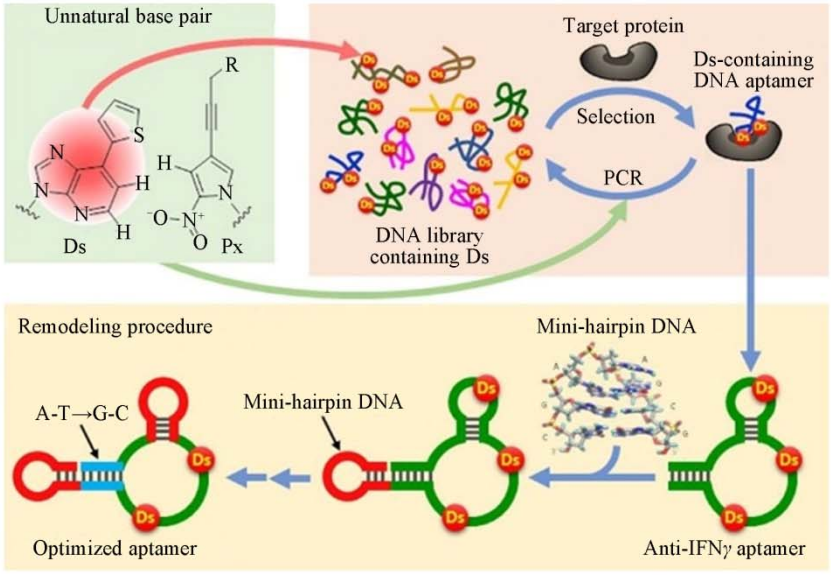

Fig.2 Synthesis and mechanism of recombinant DNA aptamers[51]

The synthesis scheme and structure of DNA aptamers based on two artificial bases Ds and Px are shown in this figure.

tumor can be controlled. Cogoi *et al.*[52] synthesized an oligonucleotide modified by nucleic acid locking(LNA) and inserted polycyclic aromatic hydrocarbons into it, which not only enhanced the nuclease resistance of oligonucleotide, but also facilitated its space folding to form g-quadruplet. They found that this oligonucleotide can inhibit the expression of KRAS in pancreatic cancer cells, thus inhibiting the proliferation and metabolism of pancreatic cancer cells, so it has a good application prospect in the treatment of pancreatic cancer.

3.2 RNA Aptamers

MicroRNA(miRNA) is an endogenous RNA that can inhibit the translation of target genes and degrade target genes. Nucleic acid aptamer-miRNA chimera can effectively inhibit the expression of miRNA, so as to achieve the effect of tumor therapy. Recently, researchers have found that RNA aptamers can also target TF, a cancer-causing transcription factor, and inhibit its transcriptional activity. Xie *et al.*[53] constructed an alncRNA by using the miRNA sponges covalently modified RNA aptamers based on the method of synthetic biology. This synthetic alncRNA can target TF and miRNA of bladder cancer cells and simultaneously inhibit TF transcription and post-transcriptional regulation of miRNA, thus effectively treating bladder cancer. Therefore, this RNA aptamer-miRNA chimera significantly improves the efficiency of tumor therapy and is expected to achieve more efficient bladder cancer therapy.

Nucleic acid aptamers often promote tumor cell death by targeting tumor cells. In addition, nucleic acid aptamers can also stimulate the immune activity of immune cells by combining with immune cells, thus promoting the immune system to eliminate tumor cells. In addition, appropriate chemical modification can also enhance the stimulation of nucleic acid aptamers to the immune system, thus improving the anti-tumor effect. McNamara *et al*.[54] effectively activated immune T cells in mice by using biotin to modify the 5′ end of the m12—23 aptamer(an RNA aptamer). They found that the aptamer was able to target the activation of 4-1BB receptors on the surface of T cells, which significantly increased the stimulation of nucleic acid aptamers on immune T cells. In addition, this aptamer can be synthesized in large quantities by chemical means, which is expected to significantly reduce the cost of tumor treatment.

Small interfering RNA(siRNA) is a double-stranded RNA that binds to complementary mRNAs in gene transcription[55—57], preventing it from being translated further and silencing the transcriptional genes. RNA-siRNA chimera is formed through the interaction between RNA, which improves the targeting ability of RNA aptamers, and then the treatment of tumor by siRNA interference. Therefore, the chimera has the advantage of more accurate drug delivery than the two RNAs alone. Dassie *et al.*[58] used prostate-specific membrane antigen(PSMA)-oncogene Plk1 chimera to treat prostate cancer. In addition, they truncated the RNA aptamers to make them easier to synthesize. Experiments showed that the optimized chimera also significantly reduced the number of prostate cancer cells in mice through systemic administration, which proved its extremely high targeting and anti-cancer ability. Because it is easy to be synthesized chemically and has good therapeutic effect, this chimera has a good application prospect in tumor therapy.

3.3 PNA

PNA is a kind of peptide nucleic acids, which is also a kind of DNA analogues[59—61]. The miR-210 can help tumor cells overcome hypoxia, so the expression of miR-210 has a certain relationship with the pathogenesis of tumor. If the function of miR-210 can be inhibited, the treatment of tumor can be realized. Gupta *et al.*[62] designed a pegylated antisense peptide nucleic acid PNA to inhibit miR-210, which was delivered *via* PLGA nanoparticles to inhibit tumor proliferation. In addition, they proposed that delivering γPNAs with modified nanoparticles enhanced their anti-tumor effect. This work provides a new approach for the targeting of miR-210 and tumor therapy.

4 Drug Delivery System Based on Artificial Nucleotide-containing Aptamers

The nucleic acid aptamer is expected to achieve accurate tumor therapy due to its high targeting and good water solubility[63,64]. Therefore, nucleic acid aptamers can be used as targeting agents in drug carriers, and there are many ways to construct drug carriers based on chemically modified nucleic acid aptamers[65—67]. Most commonly, drug carriage, such as coating, is achieved by non-covalent conjugation[68,69]. In addition, drugs or bridging agents can be chemically linked to aptamers to build drug carriers[70,71].

Passive diffusion of drugs requires carrier proteins to open channels. Drug resistance makes the carrier protein insensitive to drugs, reducing the efficiency of drug entry into cells. In addition, resistance causes over-expression of receptor proteins that transport anti-tumor drugs, allowing drugs that enter the cell to be quickly transported out of the cell. However, due to its targeting, aptamer can specifically bind receptor proteins to inhibit its over-expression, so that drugs can be effectively transferred into cells. Moreover, the aptamers do not enter the cells by passive diffusion. Therefore, nucleic acid aptamers have a good application prospect in overcoming multi-drug resistance.

We found that aptamers can participate in the construction of drug carriers together with fluorescent probes and other drugs. This drug carrier can be used to detect and treat cancer simultaneouly[72]. pRNA-3WJ(three-way junction) is made up of three short strands of RNA, which can carry three different substances at the same time, allowing the aptamer to be connected and the drug to be loaded simultaneously. By using this three-junction structure, Pi *et al.*[73] integrated Endo28 (athioaptamer to bind specifically to human ovarian cancer cells), fluorescent probe and doxorubicin on a platform to build a drug carrier that can target ovarian cancer cells. In addition, they found that the drug carrier was able to convert the passive transport of doxorubicin into endocytosis, improving its ability to enter the cell without being blocked by the receptor protein, thereby avoiding multi-drug resistance in tumor therapy.

G3139 is an antisense oligonucleotide that can target apoptotic genes, but it is not stable in physiological environment and is easily hydrolyzed by nuclease. Ma *et al.*[74] modified G3139 with thiophosphate ester to improve its stability(Fig.3), and used mixed lipids as drug carriers to achieve tumor treatment. This mixed lipid was connected to G3139 through intermolecular interaction rather than chemical bond, and the resultant compound carrier not only enhanced the stability of G3139, but also showed no cytotoxicity. Therefore, this compound drug carrier has great application potential in tumor therapy.

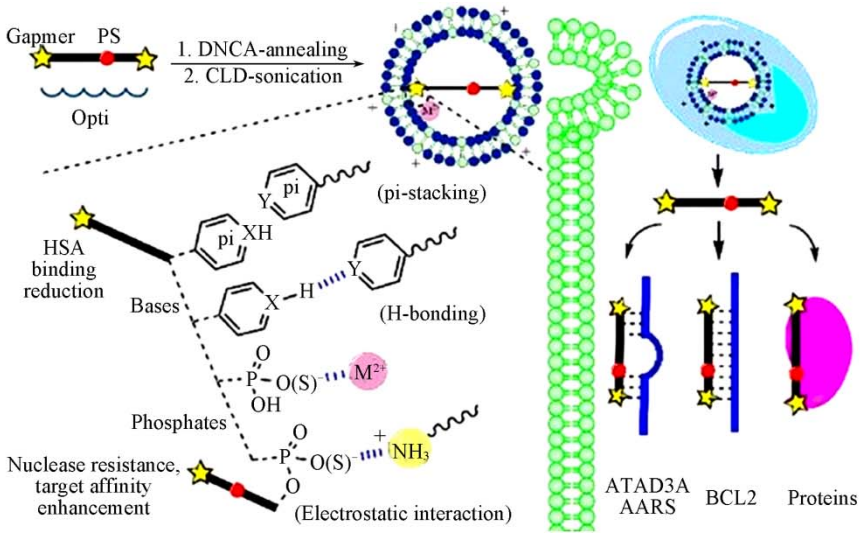

Fig.3 Schematic diagram of drug carrier's construction[74]

The construction of drug carriers requires the chemical modification of DNA aptamers first, and then the connection between aptamers and liposome through intermolecular interaction, such as hydrogen bond and electrostatic interaction.

When combine with drugs, it is possible to improve the effectiveness of cancer treatment by reducing drug resistance. Bertucci *et al.*[75] used mesoporous silica as drug carriers and loaded the anticancer drug temozolomide(TMZ), and connected it to polyarginine-peptide nucleic acid(R8-PNA) with targeted anti-tumor function through intermolecular interaction. Thus, the particles were able to deliver both R8-PNA221 and TMZ to drug-resistant glioma cells. In addition, they found that the particles could also induce apoptosis in TMZ-resistant tumor cells, demonstrating that two drugs could achieve more effective anti-tumor therapy than a single one.

5 Conclusions

This paper reviews recent applications of artificial modified nucleotides in tumor therapy, including tumor therapeutic agents and delivery of targeted drugs. From these applications, it can be seen that the current researches focus on optimizing the chemical function of nucleic acid aptamers through a variety of chemical modifications, and constructing targeted drug carriers to load existing anti-tumor drugs for collaborative therapy. However, the current research on artificial modified nucleotides is still limited by the few types of artificial nucleotides and the unclear anti-tumor mechanism. With the further development of the research, artificial modified nucleotides will have broader application prospect in the field of tumor therapy.

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