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Akira Murakami *Editor*

Nucleic Acid Drugs

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Akira Murakami
Editor

Nucleic Acid Drugs

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Dedicated to Dr. Paul O. P. Ts'o
Dr. Paul C. Zamecnik
Dr. Alan M. Gewirtz

Preface

Two giants of nucleic acid drug (NAD) research, Dr. Paul O.P. Ts'o of the Johns Hopkins University and Dr. Paul Zamecnik of Harvard University, passed away in 2009. Both left big footprints on the path toward the discovery of NAD-based therapeutics. One of the Professor Ts'o's deepest imprints was the publication of his first paper on antisense technology, which described the synthesis of trinucleotides with triphosphate linkages to regulate tRNA function. He was well aware of the potential application of the technology, when predicting that the anionic plasma membrane may prevent the cellular uptake of NADs. He also realized that the removal of negative charges from NADs was essential for molecular design, and may increase the stability of hybrids formed between NADs and target RNAs. Ultimately, these effects were found to be limited, but the concept is highly appreciated. Dr. Ts'o then developed oligonucleoside methylphosphonate (OMP) which consisted of methylphosphonate linkages in place of the phosphodiester linkages in DNA. OMPs are resistant to cellular nucleases. Moreover, these compounds are not negatively charged, which facilitates their cellular uptake. However, stereoisomers of methylphosphonate linkages destabilized the hybrids between OMPs and the target RNAs. Thus, OMPs are disappearing from the front line of NAD research. On the other hand, the methylphosphonate linkage, when introduced at either of both termini of NADs, has found continued use in enhancing the exonuclease resistance of NADs. Dr. Zamecnik used oligodeoxyribonucleoside phosphodiesters (ODNs) to specifically suppress the replication of Rous sarcoma virus. His experiments, using inactivated culture media, validated antisense technology and indicated a promising future for NADs. In the early 1980s, Dr. Paul Miller's group at the Johns Hopkins University confirmed that the antisense mechanisms regulate mRNA function. In the mid-1980s, Dr. Matsukura's group at the National Institutes of Health (NIH; USA) made the striking discovery that oligonucleoside phosphorothioate compounds (OPTs) could efficiently inhibit HIV replication. Dr. Fritz Eckstein from the Max Planck Institute in Göttingen, Germany, developed OPTs as innovative tools for studying the mechanisms of enzyme action. These reports attracted enormous attention by researchers working in diverse fields of life science and initiated intensive research aimed to develop

NADs as therapies for HIV/AIDS. Although inhibition of HIV replication did not result solely from antisense effects, the reports certainly impressed researchers. Because of their superior chemical, biochemical, and biological advantages, OPTs have been studied to a greater extent than other NADs and have more frequently found their way into clinical trials. OPTs, like OMPs, are mixtures of stereoisomers that can adversely affect antisense activity. It was later determined that the stereocontrolled synthesis of OPTs might produce more efficacious antisense ODNs. Dr. Wojciech Stec of the Polish Academy of Science developed the first method for stereospecific synthesis of OPTs and reported that their biological characteristic varies depending on which stereoisomers is used. Unfortunately, all clinical trials have utilized mixtures of stereoisomers.

Based on the efforts of these pioneers in NAD research, various NAD concepts have been presented. In this review issue, various types of NADs are introduced, and the recent status of NAD research is summarized by leaders in the field. This issue focuses on the following six aspects of NAD: (1) antisense oligonucleotides, (2) decoy DNA, (3) CpG-oligonucleotides, (4) aptamers, (5) NAD delivery, and (6) therapeutic applications. They have been involved in innovating new, particularly noteworthy synthetic routes for stereocontrolled synthesis of OPTs.

In Chap. 1, the basic concept of antisense and siRNA strategies is summarized by Dr. Yano of Nippon Shinyaku Co. The antisense strategy can be regarded as the striking NAD concept that was logically innovated in the mid-1970s. Originally, oligonucleotides complementary to mRNAs were thought to physically block translational steps in gene expression resulting in the blocking of protein synthesis. In the mid-1980s, it was reported that hydrolysis of the target mRNA by RNaseH was involved in the mechanism of antisense effects. RNaseH activity induced by certain types of NAD can drastically influence the effect of antisense molecules. Therefore, the type of NAD must be carefully chosen depending on the function of the target. For example, controlling alternative splicing by antisense molecules requires NADs that do not induce RNaseH activity. BNA (LAN) and morpholino-oligonucleotides are among the most suitable NADs for this purpose and have been used clinically to treat muscular dystrophy based on the exon-skipping concept.

In the mid-1990s, Fire and Mellow independently made the remarkable discovery that small concentrations of double-stranded RNAs suppress the function of mRNA by a quite small dose in a highly sequence-specific manner. This technique, RNA-interference (RNAi), serves as the basis for gene silencing protocols, and siRNAs are being studied intensively with regard to their potential as effective NADs. However, there are three major limitations. First, dsRNA is easily hydrolyzed in serum condition, and to induce RNAi by the molecules, they must acquire nuclease resistance. Thus, certain chemical modifications on siRNA are required. Second, siRNA (dsRNA) has to be transported into the target cells. Until date, several types of dsRNA analogs have been developed to address these problems, but certain drug stabilization materials and drug delivery systems (DDSs) should be innovated. This aspect is also discussed in Chap. 5. Third, it is quite difficult to chemically synthesize long RNAs for siRNA or miRNA studies. Dr. Yano's group have made great development in this aspect. It is important to note that it took a long

time to develop antisense drugs after the discoveries of Dr. Ts'o and Dr. Zamecnik. The discovery of the ability of siRNAs to specifically target mRNAs was made almost 25 years after RNA silencing was first described, and it may take as long or longer to develop efficacious siRNA-based drugs.

Chapter 2 reviews the decoy strategy. As the details of mechanisms of gene transcription were revealed, specific binding of transcription factors to dsDNA in a sequence-specific manner attracted researchers' attention with regards to the development of NADs for targeting this critical process. Thus, the function of certain transcription factors (TFs) could be regulated by dsDNA by binding to their DNA-recognition site. This is termed the dsDNA decoy mechanism. The advantage of the decoy strategy is that decoy molecules can potentially regulate gene expression at specific stages in gene regulation, and therefore, they can target physiological processes such as cell differentiation and proliferation. Dr. Morishita's group at Osaka University is a leader in pursuing the decoy strategy for drug development. They developed a nonchemically modified decoy DNA, the ribbon type decoy, and chimera decoy. Recently, a special AT-rich sequence binding protein-1 (SATB-1) was found to be a key factor in the regulation of various transcriptional processes. SATB-1 binds to chromatin in a sequence-specific manner, and this binding triggers the looping out of the double-stranded region, which facilitates the recruitment of TFs. This process may be a suitable target of the decoy approach. Decoy activity requires the accumulation of decoy molecules in nuclei; therefore, a suitable DDS is essential for clinical application.

In Chap. 3, immunostimulatory properties of synthetic oligonucleotides are reviewed by Dr. Agrawal of Idera Pharmaceuticals. In the 1960s, PolyI/PolyC was found to be immunostimulatory and was applied as a chemotherapeutic reagent. During studies of antisense strategy, some curious phenomena were observed. Some oligonucleotides containing a specific sequence, CpG (cytosine-phosphate-guanosine), stimulated immune functions of ODN-treated cells. Later, these phenomena were explained by the natural immunity imparted by Toll-like receptors (TLR 7, 8, 9). It has been revealed that single-stranded RNA is the ligand for TLR7 and TLR8, and that bacterial and viral DNA containing a CpG-motif are ligands for TLR9. Based on these findings, oligonucleotide-based antagonists against TLRs were developed. Dr. Agrawal's group now evaluating for candidates in clinical trials.

In Chap. 4, Dr. Nakamura's group at the University of Tokyo reviews the aptamer strategy with emphasis on RNA technology. The concept of the aptamer was first presented in the late 1980s, when it was reported that a DNA fragment could bind to a unique protein in a highly sequence-specific manner. Thus, thrombin activity is effectively inhibited by ODNs selected by the systemic evolution of ligands by exponential enrichment (SELEX) protocol from a pool of ODNs representing a huge library of unique sequences. This finding was remarkable for a number of reasons. The interaction between thrombin and ODN is thought to be the result of specific interactions involving the tertiary structures of both components. The discovery of aptamers was achieved by combinatorial chemistry, and can be compared to the fable "Finding a needle in a haystack."

This time-consuming protocol enabled the development of the novel NAD drug, Macugen. Variations of this concept are now being widely applied.

Dr. Nakamura's group has developed RNA-aptamers focusing on TNA's remarkable conformational plasticity.

Dr. Kataoka of the University of Tokyo reviews recent developments in polymer-based DDS technology in Chap. 5. As stated above, the optimization of NAD delivery systems is a key to a successful development of therapeutics; however, this goal has not been realized since the time Dr. Ts'o focused on the subject in the early 1970s. In this review, the challenges to the long-lasting issue are summarized with respect to polymer science, and the developments of polymer-based NAD carriers by Dr. Kataoka's group are introduced. His group has defined numerous factors required for effective delivery of NADs into cells and for the effective release of NADs into the cytosol.

In Chap. 6, recent developments in NAD clinical trials are reviewed by Dr. Gewirtz' group at the University of Pennsylvania. NADs have been evaluated in clinical trials for 40 years. Recent trials have been cited on the homepage of the NIH. In 2011, more than 300 NAD clinical trials were filed. In this review, recent clinical trials are introduced and reviewed in detail, and it should convince readers that NADs have significant therapeutic potential. To our deepest regret, one of the pioneers in this field, Dr. Alan M. Gewirtz, passed away in November 2010. However, his great achievements in the clinic and laboratory will be appreciated by young researchers who chose to pursue his research passions.

The overview of developments in NAD technology presented here should convince us that NADs could indeed be magic bullets to cure and control many diseases. Although number of trials were forced to withdraw at both preclinical and clinical stages, there must be ways to reach the final goal. I sincerely hope that the readers of this issue, mainly young polymer scientists and those working in other fields, will appreciate the encouraging messages for NAD research and development by our contributing authors.

Kyoto, Japan Akira Murakami

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New Antisense Strategies: Chemical Synthesis of RNA Oligomers

Junichi Yano and Gerald E. Smyth

Abstract In this chapter, we introduce our recent work on the development of new antisense strategies in RNA synthetic technology and RNA drug discovery. In the first part of the chapter, we briefly introduce selected chemical modifications of antisense oligomers and recent developments in antisense RNA. In the second part, we describe our research on RNA, including synthetic approaches to stereodefined backbone-modified oligoribonucleotides with chiral linkages, and the development of a method for the synthesis of RNA based on the use of 2-cyanoethoxymethyl (CEM) as the 2′-hydroxyl protecting group. The CEM method has been applied to the synthesis of single and double short hairpin RNA, pre-microRNA, and designed mRNA molecules up to 170 nucleotides in length, which were physicochemically identified and shown to be biologically active. We hope that the work presented here will contribute to the pushing forward of the frontiers of knowledge and understanding in RNA biology and RNA drug discovery.

Keywords 2-Cyanoethoxymethyl · Artificial mRNA · Double short hairpin RNA · RNA synthesis · Stereospecific modification

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Abbreviations

ACE	Bis(2-acetoxyethoxy)methyl
Ap^bA	Diadenosine 3′,5′-boranophosphate
Bcl-2	B-cell lymphoma 2
BH_3-ODN	Boranophosphate oligodeoxynucleotide
BH_3-ORN	Boranophosphate oligoribonucleotide
BNA	Bridged nucleic acid
CEE	2-Cyanoethoxyethyl
CEM	2-Cyanoethoxymethyl
CEM-Cl	Chloromethyl ether
DMTr	4,4′-Dimethoxytrityl
dshRNA	Double short hairpin RNA
dsRNA	Double-stranded RNA
ELISA	Enzyme-linked immunosorbent assay
FDA	Food and Drug Administration
GLP-1	Glucagon-like peptide-1
HOX	Homeobox
HPLC	High-performance liquid chromatography
IFN	Interferon
LNA	Locked nucleic acid
miRNA	micro RNA
mRNA	Messenger RNA
NMR	Nuclear magnetic resonance
NOE	Nuclear Overhauser effect
nP1	Nuclease P1
nt	Nucleotide(s)
ODN	Oligodeoxynucleotide
poly(A)	Polyadenylic acid
poly(U)	Polyuridylic acid
pre-miRNA	Precursor micro RNA

PS-ODN	Phosphorothioate oligodeoxynucleotide
PS-ORN	Phosphorothioate oligoribonucleotide
RISC	RNA-induced silencing complex
RNAi	RNA interference
RNase H	Ribonuclease H
RT-PCR	Real-time polymerase chain reaction
shRNA	Short hairpin RNA
siRNA	Small interfering RNA
svPDE	Snake venom phosphodiesterase
TBDMS	2′-*O*-*t*-butyldimethylsilyl
TEM	2-(4-Tolylsulfonyl)ethoxymethyl
TOM	Triisopropylsilyloxymethyl
UTR	Untranslated region

1 Introduction

The antisense concept is founded on an understanding of the sequence-specific binding of an oligonucleotide to complementary RNA targets by Watson–Crick base-pairing. The goal of antisense technology is to introduce into the cell a synthetic oligonucleotide that hybridizes with a preselected target RNA, leading to cleavage or disablement of the target RNA for research or therapeutic purposes. The history and current state of the field are well described elsewhere, such as in the multiauthor volumes *Antisense Drug Technology* [1, 2] and *Antisense Research and Applications* [3, 4].

Historically, attention was first focused on antisense oligodeoxynucleotides (ODNs), usually chemically modified single-stranded ODNs 18–21 nucleotides (nt) in length that hybridize with mRNA to form a DNA–RNA heteroduplex. The mRNA strand of the heteroduplex is not only physically blocked so that it cannot be translated, but it is also specifically cleaved by the endonuclease ribonuclease H (RNase H), leading to the suppression of gene expression [5]. In 1978, Zamecnik and Stephenson [6, 7] proposed the therapeutic use of synthetic oligonucleotides on the basis of their finding that Rous sarcoma virus replication in chicken fibroblast tissue-culture cells can be inhibited by a synthetic 13-nt ODN complementary to the 3′ and 5′ ends of the viral 35 S RNA. Antisense nucleic acids have been under investigation ever since, but progress in their therapeutic application has been slow, and it was not until 1998 that the first antisense drug was approved by the Food and Drug Administration (FDA). This was Fomivirsen (Vitravene), developed by Isis Pharmaceuticals for the treatment of cytomegalovirus retinitis and delivered by intraocular injection. Another antisense ODN, G3139 (Genasense; Oblimersen), developed by Genta to target the antiapoptotic protein B-cell lymphoma 2 (Bcl-2), progressed to Phase III clinical trials in several cancers but has not received FDA approval. To date, no further antisense drugs have received FDA approval.

For therapeutic applications, antisense oligonucleotides should have high resistance to degradation by nucleases, excellent cell membrane permeation characteristics, and high affinity for the target RNA [8]. At the same time, they should not bind to nontargeted nucleic acid sequences or undergo unintended interactions with proteins or other biological molecules. To develop oligonucleotides with these properties, it has been found necessary to introduce chemical modifications, mainly at the sugar moiety and the phosphodiester linkage. The first generation of modified antisense ODNs was prepared by replacing an oxygen atom of the phosphodiester backbone with a methyl group (to give methylphosphonates; see Sect. 2.1.1), a sulfur atom (to give phosphorothioates; see Sect. 2.1.2), or a 3′ amino group (to give phosphoramidates) [9]. A second generation of antisense oligonucleotides was developed by replacing the 2′-hydroxyl hydrogen atom of oligoribonucleotides with methyl or methoxyethyl groups (Sect. 2.2.1) or the entire 2′-hydroxyl group with a fluorine atom [9, 10]. Other types of antisense oligonucleotide and oligonucleotide mimic have also been developed, such as bridged nucleic acids (BNAs) (Sect. 2.2.2), peptide nucleic acids, and phosphorodiamidate morpholino oligomers (morpholinos). Oligonucleotide analogs that retain a negative charge on the phosphate internucleotide linkage support RNase H activity. Replacement of a nonbridging phosphate oxygen generates oligomers with chiral phosphorus centers that generally consist of mixtures of diastereomers with poorly defined properties. Yet, their stereospecific synthesis allows the selection of the favorable diastereomer (e.g., the more nuclease-resistant or the more active diastereomer), an approach that might be useful in the design of antisense oligonucleotides (see Sect. 5).

In the early 1980s, ribozymes, which are ribonucleic acids with catalytic activity such as the cleavage of RNA itself and self-splicing, were reported by Cech and Bass [11] and Altman [12]. Ribozymes were quickly recognized to have therapeutic potential [13], and some progress has been made in this area [14–16]. However, these efforts have yet to come to full fruition, possibly because of the intrinsically low RNA cleavage activity of ribozymes in vivo. Then in 1998 Fire, Mello and coworkers [17] published a key paper on the phenomenon of gene silencing by RNA interference (RNAi) in the nematode worm *Caenorhabditis elegans*. Generalized to eukaryotes [18], this work has given a great impetus to the development of nucleic acid drugs [19]. Tuschl's group [20] showed that small interfering RNA (siRNA), i.e., double-stranded RNA (dsRNA) of 21–23 nt in length, functions in RNAi-mediated gene silencing in mammalian cells, including human cells. siRNA is under intensive investigation as a potential new class of therapeutic agent with a high specificity for its molecular target [21–23].

In the intervening years, many classes of small functional noncoding RNAs have been discovered [24, 25], some of which have therapeutic relevance. One in particular, microRNA (miRNA), is a family of endogenous single-stranded RNA molecules approximately 22 nt in length that play important regulatory roles in animals and plants by targeting mRNA for translational repression or cleavage [26–28]. miRNAs have been implicated in the pathogenesis of several classes of human disease, including cancer, neurodegenerative disorders, and viral and

metabolic diseases [29], and are under investigation as another new class of therapeutic target for antisense drugs [29, 30].

For development as therapeutic agents, oligonucleotides must not only show high efficacy in humans and have a high margin of safety, but they must also be capable of large-scale, high-quality, low-cost production, demands that have been difficult to combine for nucleic acids. An important consideration in antisense research is the development of systems to deliver oligonucleotides to the appropriate tissues and organs and, where necessary, to assist their entry into the cell. Antisense DNA oligomers have been at the forefront of nucleic acid drug development mainly because their synthesis and chemical modification were easier than for RNA oligomers. Now, however, DNA/RNA chimeras and RNA oligomers are playing an increasingly important role in oligonucleotide therapeutics. RNA has the advantage that it encompasses a variety of therapeutic approaches besides antisense, including aptamers, ribozymes, and RNAi.

In the following sections, we briefly review selected chemical modifications of antisense oligomers (Sect. 2) and recent developments in antisense RNA (Sect. 3). We then describe our own work on RNA, including synthetic approaches to stereodefined backbone-modified oligoribonucleotides with chiral linkages (Sect. 5) and the development of an RNA synthetic method based on the use of 2-cyanoethoxymethyl (CEM) as the 2′-hydroxyl protecting group (Sect. 6). The CEM method (Sect. 6.1) allows the ready synthesis of highly pure RNA oligomers in a yield comparable to that obtained in DNA synthesis. The method has been applied to the synthesis of precursor microRNA (pre-miRNA) (Sect. 6.2), single and double short hairpin RNA (dshRNA) (Sect. 6.3), and designed mRNA molecules up to 170 nt in length (Sect. 6.4).

2 Selected Antisense Modifications

2.1 Backbone Modifications

2.1.1 Methylphosphonates

To improve the uptake of oligonucleotides by cells as well as extend their biological half-life, Ts'o, Miller and coworkers [31] eliminated the negative charge on the internucleotide phosphate bridge by replacing a negatively charged phosphate oxygen atom not involved in the bridge by the neutral and sterically undemanding methyl group. The resulting methylphosphonates [32] are an early example of neutral oligonucleotide analogs. The methylphosphonate diester linkage is chiral, and *R*p oligomers form more stable complexes with natural RNA oligomers than do *S*p or racemic oligomers [33]. (*R*p and *S*p refer to the configuration of the phosphorus atom as determined by the Cahn–Ingold–Prelog priority rules.) Methylphosphonates are taken up by mammalian cells in culture and have

high nuclease resistance. This modification also promotes the formation of stable duplexes. However, methylphosphonates, as well as other modified oligonucleotides with uncharged internucleotide linkages, do not support RNase H activity. For this reason, and also because of their poor solubility, they are not considered desirable for most oligonucleotide drug applications. Nevertheless, this modification may find a niche as a site-specific modification to improve nuclease resistance.

2.1.2 Phosphorothioates

In phosphorothioate oligodeoxynucleotides (PS-ODNs), a nonbridging oxygen atom of the phosphodiester linkage is replaced by a sulfur atom, with the negative charge being distributed unsymmetrically and located mainly on sulfur. Phosphorothioates are among the most obvious and therefore one of the earliest-used nucleic acid analogs [34], whose antiviral effect was described in 1970 by De Clercq et al. [35]. PS-ODNs have the advantages that they (1) are easily prepared, (2) are stable to base-catalyzed hydrolysis, (3) are highly water-soluble, (4) are resistant to nucleases, (5) show good binding to plasma proteins, which prevents their rapid renal excretion and assists in their in vivo delivery, and (6) support RNase H activity. These properties make phosphorothioates exceptionally suitable for use in antisense technology. Like the methylphosphonate linkage, the phosphorothioate diester linkage is chiral, so that phosphorothioate oligomers are complex mixtures of diastereoisomers with poorly defined melting temperatures. For this reason, as far as the recognition of sequence specificity is concerned, accurate target validation is difficult. Nevertheless, phosphorothioates remain the most commonly used chemical modification in oligonucleotide therapeutics. Chemical approaches to the synthesis of diastereomerically pure phosphorothioates are described in Sects. 5.1 and 5.2.

2.1.3 Boranophosphates

In boranophosphate oligonucleotides, a nonbridging phosphodiester oxygen is replaced by a borane (BH_3^-) group [36, 37], so that the boranophosphate linkage, like the methylphosphonate and phosphorothioate linkages, is chiral, and boranophosphate oligomers are mixtures of diastereomers. Boranophosphates are highly water-soluble and the boranophosphate linkage is more nuclease-resistant than the natural phosphodiester linkage. Boranophosphate oligodeoxynucleotides support RNase H activity [38], while boranophosphate-modified siRNA is reported to be slightly more active than unmodified siRNA in vitro [39]. Although boranophosphates have not yet found application in the development of oligonucleotide drugs, the potential of this modification continues to be explored. Our chemical approach to the stereospecific synthesis of boranophosphates is described in Sect. 5.3.

2.2 Sugar Modifications

2.2.1 2′-O-Modification

Oligonucleotides containing alkyl modifications at the 2′ position of the ribose represent the second generation of antisense oligonucleotides. 2′-*O*-Methyl, a naturally occurring modifying group, and 2′-*O*-methoxyethyl are important members of this class [40–42]. Because the 2′-hydroxyl group is alkylated, there is no possibility of isomerization of the internucleotide linkage, and the alkylated oligoribonucleotides are stable to base-catalyzed hydrolysis. Modification of the 2′-hydroxyl group of the ribose also overcomes RNA's susceptibility to nuclease degradation. For example, 2′-*O*-methyloligoribonucleotides are resistant to a wide range of nucleases. 2′-*O*-Methyloligoribonucleotides also form stable duplexes with complementary, unmethylated natural oligoribonucleotides, but, as expected, the duplexes do not support RNase H activity. Non-naturally occurring 2′-*O*-alkyl ethers of ribonucleosides, such as 2′-*O*-allyl and 2′-*O*-ethyl oligoribonucleotides [43–45], have also been investigated. 2′-*O*-Methoxyethyl-modified nucleotides have found application in chimeric antisense oligonucleotides known as gapmers [10], in which runs of 2′-O-modified ribonucleotides at the ends of the oligonucleotide increase nuclease resistance and binding affinity for the target RNA, while deoxyribonucleotides in the interior of the oligonucleotide elicit RNase H activity.

2.2.2 Bridged Nucleic Acids

BNA are oligonucleotides containing 2′,4′-bridged nucleotides. An example is 2′,4′-methylene bridged nucleic acid, also known as locked nucleic acid (LNA) [46–49]. Introduced in 1998, this modification was intended to improve the affinity of binding to complementary DNA and RNA oligonucleotides. LNA nucleotides adopt the A-type structure by locking the furanose ring in the north conformation (C2′-*exo*–C3′-*endo* pucker) preferred by A-form RNA duplexes. The incorporation of one or more LNA nucleosides into antisense ODNs increases their nuclease resistance. The binding affinity of LNA to nucleic acids is indeed unprecedentedly high, though the thermodynamic explanation for this is complex. LNA:RNA hybrids do not support RNase H activity; for recruitment of RNase H, DNA segments must be included in the design of the LNA. Several LNA analogs with therapeutically useful properties, such as amino-LNA, thio-LNA, and α-L-*ribo*-LNA, have also been developed. LNA-containing ODNs have been shown to be pharmacologically active in multiple tissues and species by multiple administration routes, with both mRNA and miRNA as the target. LNA drugs are under development for the treatment of cancer [50]. An LNA-modified oligonucleotide complementary to miR-122 has been shown to lead to long-lasting suppression of hepatitis C virus in chimpanzees [51] and is the first miRNA-targeting RNA drug to enter clinical trials. Imanishi and coworkers [52] have been pioneers in the design and synthesis of a number

of 2′,4′-BNA-modified siRNA molecules, one of which is 2′,4′-aminomethylene BNA, a recently developed bridged nucleic acid that shows excellent nuclease resistance and slightly better target binding affinity than 2′,4′-methylene BNA (LNA).

3 Antisense RNA Oligomers

Since the work on RNAi in *C. elegans* in 1998 [17, 53], RNA research focused on the analysis of new classes of noncoding RNA has blossomed, and it has had a major impact in biology, medicine, and drug discovery. Small RNA molecules, especially siRNA and miRNA, have assumed great importance in drug discovery as potential therapeutic agents or targets. siRNA and miRNA are closely related in that they are about the same length and can function in similar biological pathways that lead to the degradation of mRNA. The common feature of these pathways is the recognition of target RNA sequences by the antisense strand after its incorporation into the RNA-induced silencing complex (RISC), which mediates the RNAi effect [54]. However, siRNA and miRNA differ in their biogenesis: whereas siRNA is derived from longer dsRNA, miRNA is transcribed from the genome. siRNA and miRNA also differ in their normal biological function: whereas siRNA generally acts to degrade foreign RNA, miRNA acts as a post-transcriptional regulator by binding to complementary sequences mainly in the 3′ untranslated regions (UTRs) of mRNA transcripts, usually resulting in gene silencing [26, 55].

miRNA directly influences the expression of a wide range of gene types and plays an important part in growth, development, cell death, and differentiation. Over one-third of human genes are predicted to be conserved miRNA targets [56]. Currently, 922 human miRNAs are annotated in version 14 of the Sanger miRBase [57–60], and the number of known miRNAs is steadily increasing [61]. Several human diseases have been linked to a dysfunction in an miRNA pathway or to miRNA dysregulation, which may lead to inadequate control of gene expression [62–64]. Although one miRNA targets many genes, modulation of miRNA function could be of therapeutic value. An antagonist approach to the modulation of miRNA is based on the introduction into cells [29] or whole animals [65] of synthetic anti-miRNA oligonucleotides of about 22 nt known as antagomirs, which are complementary to a given miRNA sequence and can specifically inhibit miRNA function. Antagomirs can be thought of as a new category of antisense nucleic acid to block miRNA binding to its target mRNA. An example is the antagomir described by Krützfeldt et al. [65], which was modified with uniform 2′-O-methylated nucleotides and terminal phosphorothioate linkages and conjugated with cholesterol. This antagomir produced specific, efficient, and long-lasting silencing of the endogenous miRNA miR-122 in mice 24 h after administration. The Isis group [66] showed that blocking miR-122 in normal mice with a 2′-*O*-methoxyethyl phosphorothioate oligonucleotide resulted in reduced plasma cholesterol levels, increased hepatic fatty acid oxidation, and a decrease in hepatic

fatty acid and cholesterol synthesis rates, so that miR-122 could be an attractive therapeutic target for metabolic disease. The fact that one miRNA targets many genes could be turned to therapeutic advantage by making it possible to design small multiple-target artificial RNAs to simultaneously target a predetermined set of genes [67]. In addition, it has been reported that circulating miRNAs exist in the blood of cancer patients [68, 69], so that it might be possible to use miRNA as a new kind of disease biomarker. All of this illustrates the therapeutic relevance of miRNA.

Despite the fact that the initial therapeutic promise of antisense ODNs remains unfulfilled, research to date on antisense ODNs, including their chemical modification, has greatly contributed to the development of nucleic acid medicines based on RNA oligomers, which may in fact prove to be more suitable than ODNs for therapeutic applications. This is one reason that we have been focusing our research efforts on RNA. One line of research in our laboratory has led to the development of an improved method of RNA synthesis that has been extended to the production of oligomers up to 170 nt in length. This method, introduced in Sect. 6, is expected to contribute to the supply of long-chain RNA necessary for biological research and for diagnostic and therapeutic purposes, e.g., for investigating the mechanism of pre-miRNA processing and for the detection of biomarkers, as well as providing a chemical synthetic approach to mRNA. Our synthetic method can also be scaled up for the synthesis of shorter RNA molecules of therapeutic interest, such as short hairpin RNA (shRNA), for clinical trials and industrial production.

4 siRNA Versus Antisense DNA Oligomer

On entering the field of RNA drug development, we compared the in vitro and in vivo activity of an siRNA and an antisense ODN, both targeting Bcl-2 mRNA. The siRNA, B717, was composed only of natural nucleotides, whereas the antisense ODN was a phosphorothioate oligomer with the same sequence as G3139. As illustrated in Fig. 1, siRNA is a 21–23-nt dsRNA oligomer, whereas antisense DNA is a 21-nt single-stranded oligomer. Their gene-silencing mechanisms are different in that siRNA directs the cleavage of the target mRNA by RNAi in the cytosol, whereas antisense DNA blocks translation by forming a double-stranded hybrid with the complementary mRNA sequence, and also triggers the RNase-H-mediated degradation of mRNA in the nucleus.

4.1 Gene-Silencing Effect

In A549 cells (a human lung carcinoma cell line), B717 siRNA at a concentration of 100 nM almost completely suppressed the expression of Bcl-2 protein for 3 days (Fig. 2). Antisense DNA at ten times the concentration (1 μM) showed a weaker effect at all time points. Even though the antisense DNA was rendered nuclease-resistant by phosphorothioate modification, the duration of its activity was no longer

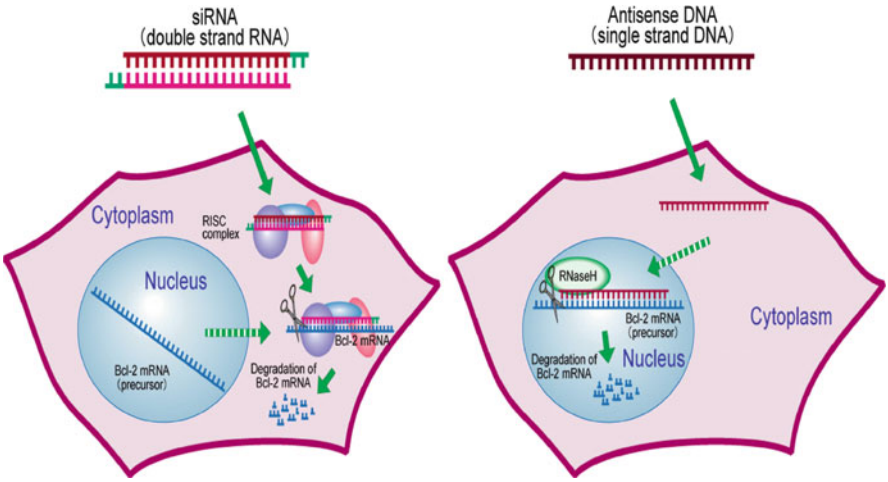


Fig. 1 Mechanisms of action of siRNA and antisense DNA

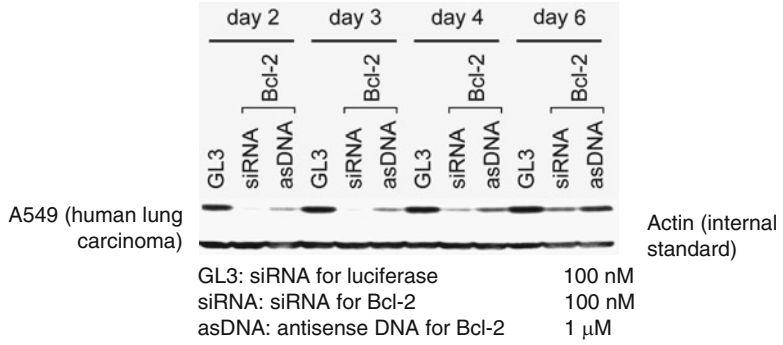


Fig. 2 Effect of siRNA on Bcl-2 protein levels in A549 cells. GL3 sense strand, 5′-CUUACGCUGAGUACUUCGAdTdT-3′; antisense strand, 5′-UCGAAGUACUCAGCGUAAG-3′. B717 (siRNA for Bcl-2) sense strand, 5′-GUGAAGUCAACAUGCCUGCdTdT-3′; antisense strand, 5′-GCAGGCAUGUUGACUUCACdTdT-3′. The negative control was the luciferase-targeting siRNA GL3. Adapted from [71]

than that of siRNA. In this experiment, a cationic liposome, LIC-101 [70, 71], was used to promote the cellular uptake of both the antisense DNA and the siRNA.

4.2 Antiproliferative Effect

In A549 cells and two other human carcinoma cell lines, A431 and MDA-MB-231, B717 siRNA showed a dose-dependent antiproliferative effect (Fig. 3) [71]. In contrast, the effect of antisense DNA was not significantly different from that of the complementary sense sequence in any of the cell lines. Thus, the antisense DNA did

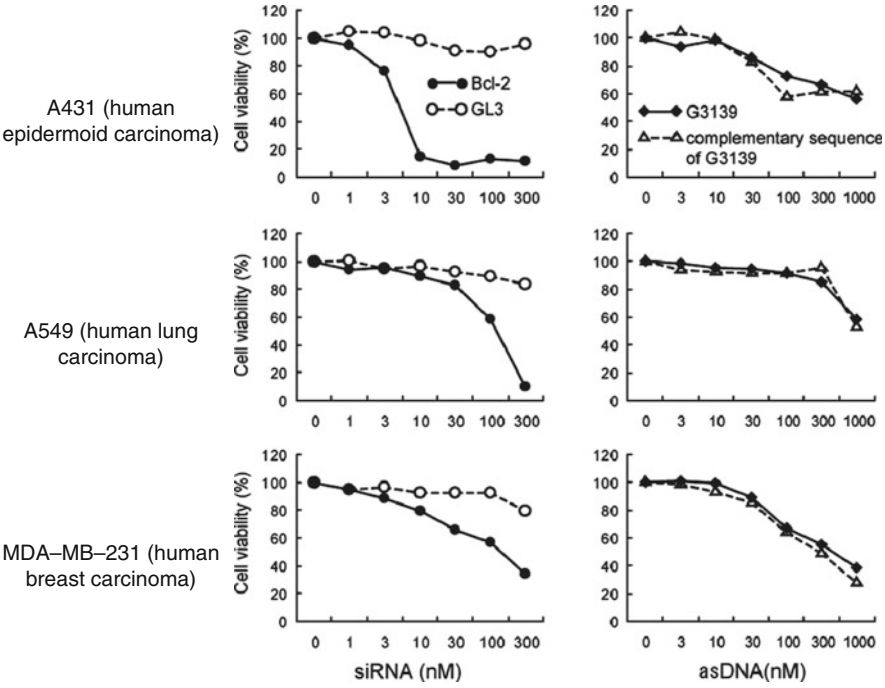


Fig. 3 Effect of siRNA and antisense DNA on the growth of human carcinoma cell lines. A431, A549, and MDA-MB-231 cells were treated with oligonucleotide/LIC-101. The antiproliferative activity of oligonucleotide/LIC-101 after 6 days was assessed by tetrazolium dye metabolic assay. *Left:* Cells were treated with B717/LIC-101 (*filled circles*) or GL3/LIC-101 (*open circles*). *Right*: Cells were treated with antisense DNA/LIC-101 (*filled triangles*) or sense DNA/LIC-101 (*open triangles*). The negative control was GL3 [71]

not show obvious sequence specificity in this experiment, and at high concentrations it had a nonspecific effect on cell proliferation that appears to be attributable to its phosphorothioate modification.

In a mouse model of liver metastasis, B717 siRNA complexed with LIC-101 administered by tail vein injection almost completely suppressed the growth of HT-1080 cells, a human fibrosarcoma cell line, at a dose of 1 mg/kg (Fig. 4, left). No effect was produced by naked B717 or LIC-101 alone (Fig. 4, right). In contrast, a dose of 4.2 mg/kg was required for the same effect with antisense DNA (Fig. 4, left) [71]. We suspect that the effect observed at this high dose was nonspecific.

4.3 Summary

siRNA had at least ten times the activity of antisense DNA in vitro and at least four times its activity in vivo. The phosphorothioate modification also appeared to show a nonspecific cytotoxicity.

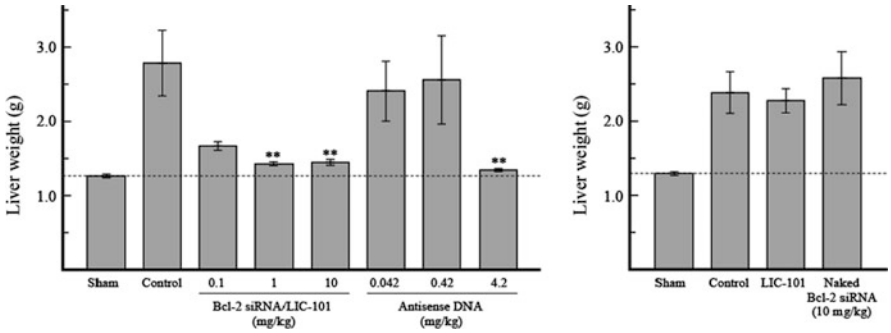


Fig. 4 Antitumor activity in a mouse model of liver metastasis. Mice were inoculated with HT-1080 cells by direct intrasplenic injection on day 0. Spleens were removed 10 min after inoculation. Each compound was administered by tail vein injection from day 4 to day 8 and from day 11 to day 15 (two 5-day cycles of daily injections). The inhibitory effect of the treatments on the increase in liver weight was evaluated on day 20. *Left*: B717 siRNA or antisense DNA complexed with LIC-101. *Right*: Naked B717 or LIC-101 alone. In sham mice, the spleen was exposed but no cells were injected. Control mice were treated with 10% (w/v) maltose solution. ** significantly different from control at $P < 0.02$

5 Stereospecific Backbone Modifications

Phosphorothioates and boranophosphates are important examples of backbone-modified oligonucleotides. Several properties of these backbone-modified oligonucleotides, such as their high nuclease resistance and their formation of stable duplexes that support RNase H activity, are superior to those of natural oligonucleotides for antisense applications. Because of the chirality of the phosphorus atom, phosphorothioates and boranophosphates synthesized by standard methods will be complex mixtures of diastereomers. Specifically, an oligomer of length n nucleotides will be mixtures of 2^{n-1} diastereomers. The physicochemical and pharmacological properties of backbone-modified oligonucleotides will generally depend on the configuration at the phosphorus center, so that an understanding of how the configuration at phosphorus affects their properties and biological activity will be crucial for their application as antisense agents. Therefore, it is important to investigate the properties of diastereomerically pure backbone-modified oligonucleotides, and we have taken steps in this direction as described in Sects. 5.1–5.3.

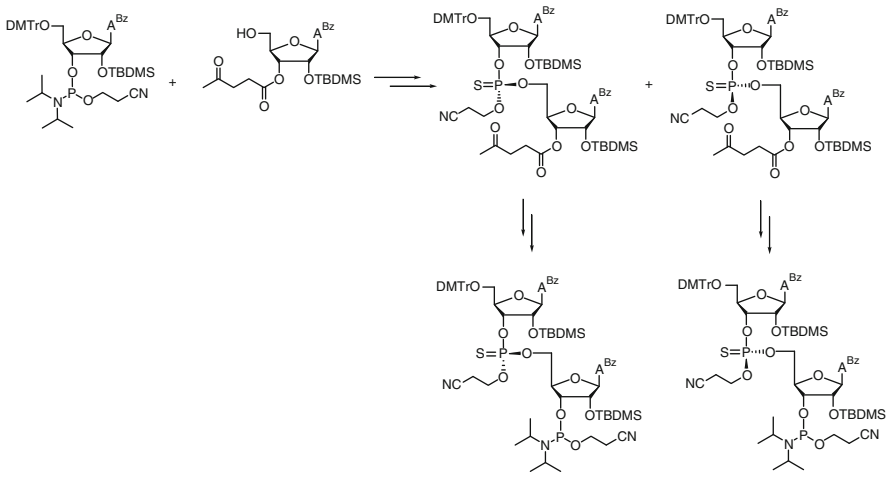
5.1 Oligoribonucleotides with Alternating Phosphorothioate Linkages

The general properties of phosphorothioate oligonucleotides were briefly described in Sect. 2.1.2. Because of the unavailability of stereodefined phosphorothioates, only a limited number of studies of the effect of the configuration at phosphorus on

the properties of phosphorothioates have been conducted [72, 73]. For example, efforts to develop a method of synthesizing stereodefined phosphorothioate oligoribonucleotides (PS-ORNs) to date have only succeeded in the synthesis of diastereomerically pure dimers or trimers [74, 75] and, with moderate stereoselectivity, 4–12-nt all-(*R*p) PS-ORNs [76, 77]. Chromatographic separation of oligoribonucleotide diastereomers containing phosphorothioate linkages has also been achieved [78].

5.1.1 Synthesis

To synthesize oligoribonucleotides containing stereodefined phosphorothioate linkages, we first prepared diastereomerically pure *S*p and *R*p phosphorothioate dimers, converted them to the amidites (Scheme 1), and used the diastereomerically pure amidite dimers in the solid-phase synthesis of a series of diastereomerically pure 21mers with alternating phosphodiester–phosphorothioate internucleotide linkages (Table 1) [79].



Scheme 1 Preparation of chiral phosphorothioate dimer amidites [79]

Table 1 Phosphorothioate-containing 21-nt oligoribonucleotides

21mer	Sequence[a]
Oligo 1	(*R*p)-5′-$A_SAA_SAA_SAA_SAA_SAA_SAA_SAA_SAA_SAA_S$AA-3′
Oligo 2	(*S*p)-5′-$A_SAA_SAA_SAA_SAA_SAA_SAA_SAA_SAA_SAA_S$AA-3′
Oligo 3	(*R*p)-5′-$U_SUU_SUU_SUU_SUU_SUU_SUU_SUU_SUU_SUU_S$UU-3′
Oligo 4	(*S*p)-5′-$U_SUU_SUU_SUU_SUU_SUU_SUU_SUU_SUU_SUU_S$UU-3′
Oligo 5	(*R*p)-5′-$A_SGG_SAG_SAA_SAU_SCA_SAA_SCA_SGA_SGG_S$dTdT-3′
Oligo 6	(*S*p)-5′-$A_SGG_SAG_SAA_SAU_SCA_SAA_SCA_SGA_SGG_S$dTdT-3′
Oligo 8[b]	5′-AGGAGAAAUCAAACAGAGGdTdT-3′

[a]Subscript "S" denotes a phosphorothioate linkage
[b]Oligo 8 is a natural oligomer

5.1.2 Nuclease Resistance

Oligonucleotides with phosphorothioate linkages showed much higher resistance to the 3′–5′ exonuclease snake venom phosphodiesterase (svPDE) and bovine serum than did the corresponding natural oligomer (Table 2). The oligonucleotide in which all of the phosphorothioate linkages were of the *S*p configuration showed particularly high nuclease resistance and serum stability.

5.1.3 Thermal Stability

Melting curves (absorbance–temperature profiles) were obtained for the complexes formed by oligo 1, oligo 2, or the natural adenosine 21mer (A_{21}) with polyuridylic acid [poly(U)], and for the complexes formed by oligo 3, oligo 4, or the natural uridine 21mer (U_{21}) with polyadenylic acid [poly(A)]. From the melting curves, the melting temperatures (T_m) of the duplexes were determined (Table 3). Oligos 1 and 3 (*R*p) had greater affinity for RNA than did natural RNA oligomers, whereas oligos 2 and 4 (*S*p) had similar or weaker affinity.

In contrast to the conventional method of sulfurization in the solid phase, the method described above can be used to synthesize phosphorothioate-containing RNA by the phosphoramidite method. Therefore, by using the highly diastereomerically pure

Table 2 Exonuclease resistance and serum stability of single-stranded oligonucleotides

	Intact oligonucleotide remaining (%)	
Oligonucleotide	Exonuclease treatment	Incubation in bovine serum
Oligo 8 (natural)	4.4	0.8
Oligo 5 (*R*p)	50	29.2
Oligo 6 (*S*p)	70	81.1

To test exonuclease resistance, the oligonucleotides were incubated with venom exonuclease phosphodiesterase I from *Crotalus adamanteus* (0.2 mU) in a total volume of 180 μL for 15 min. To test serum stability, oligonucleotides were incubated in 10% bovine serum for 30 min

Table 3 Melting temperatures of 21-nt phosphorothioate or natural oligomers complexed with poly(U) or poly(A)

Duplex	*R*/*S* configuration	T_m (°C)
Natural A_{21}:poly(U)	–	48.5
Oligo 1:poly(U)	*R*p	57.8
Oligo 2:poly(U)	*S*p	47.3
Natural U_{21}:poly(A)	–	38.0
Oligo 3:poly(A)	*R*p	44.1
Oligo 4:poly(A)	*S*p	29.7

Each 21mer at a final base concentration of 1.6 μM was mixed with an equal amount of poly(U) or poly(A) in 10 mM sodium phosphate buffer, pH 7.5, containing 0.1 M NaCl. Each mixture was heated to 70 °C and slowly cooled to room temperature, after which the absorbance at 260 nm was measured as the temperature was raised from 20 to 80 °C over a period of 2 h

phosphorothioate dimer as the synthetic unit, highly diastereomerically pure oligoribonucleotides containing alternating phosphodiester–phosphorothioate linkages can be readily prepared. The diastereomerically pure phosphorothioate dimer will also be useful for introducing stereodefined site-specific phosphorothioate modifications into oligonucleotides.

5.1.4 Summary

Synthetic diastereomerically pure phosphorothioate-containing RNA oligomers of either *S*p or *R*p configuration showed greater exonuclease resistance and serum stability than natural oligoribonucleotides did. In addition, synthetic diastereomerically pure phosphorothioate-containing RNA oligomers with the *R*p configuration showed higher affinity for complementary RNA than the natural oligomer did, whereas *S*p oligomers had similar or lower affinity.

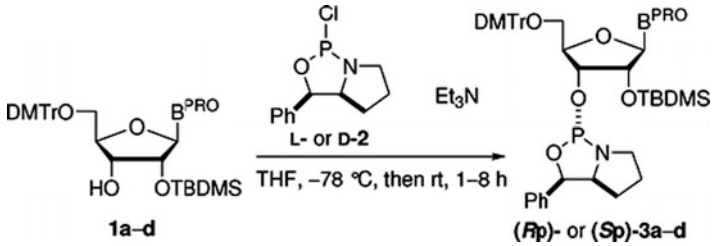
5.2 All-Phosphorothioate Oligoribonucleotides

5.2.1 Synthesis

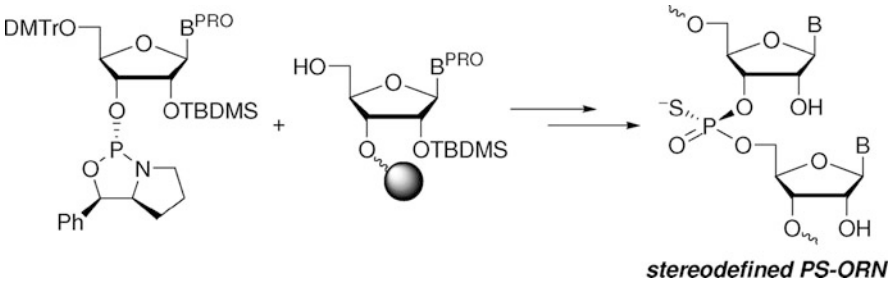
The method of synthesizing stereodefined phosphorothioate-containing oligoribonucleotides described in Sect. 5.1.1 has the limitation that it cannot be used to synthesize oligoribonucleotides containing *consecutive* stereodefined phosphorothioate linkages or fully modified stereodefined PS-ORNs. To overcome this limitation, Wada and coworkers [80] developed a method of synthesizing stereodefined PS-ORNs by using ribonucleoside 3′-*O*-oxazaphospholidine derivatives as monomer units (Schemes 2 and 3).

A proline-derived bicyclic oxazaphospholidine ring has been reported to afford the corresponding *trans*-deoxyribonucleoside 3′-*O*-oxazaphospholidines with a diastereomer ratio of >99:1 [81], and the resulting oxazaphospholidines have been successfully used in the synthesis of stereodefined PS-ODNs with a diastereomer ratio of >99:1 for each phosphorothioate linkage. Similarly, diastereomerically pure *trans*-ribonucleoside 3′-*O*-oxazaphospholidine monomers [**(*R*p)-3a–d** and **(*S*p)-3a–d**] for preparing PS-ORNs have been stereoselectively synthesized in modest-to-good yield from 2′-*O*-*t*-butyldimethylsilyl (TBDMS)-protected ribonucleosides **1a–d** and 2-chloro-1,3,2-oxazaphospholidines **L-2** and **D-2**, which are derived from L- and D-proline, respectively (Scheme 2) [81]. *trans*-**(*R*p)-3a–d** and *trans*-**(*S*p)-3a–d** configurations were assigned on the basis of the phosphorus–carbon spin–spin coupling constants ($^2J_{PC}$ values) of their oxazaphospholidine rings [81, 82].

Stereodefined PS-ORNs were manually synthesized on solid supports by using the oxazaphospholidine derivatives as monomer units according to the synthetic cycle shown in Scheme 4. Finally, all-(*R*p)-$[U_S]_9U$ (**4**) and all-(*S*p)-$[U_S]_9U$ (**5**) were



Scheme 2 Synthesis of ribonucleoside 3′-*O*-oxazaphospholidine monomers [80]

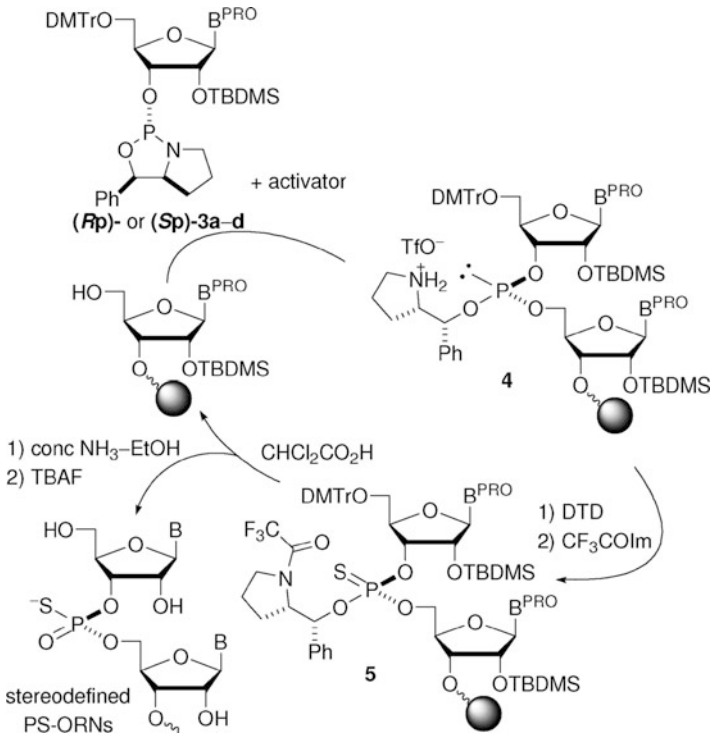


Scheme 3 Synthesis of stereodefined PS-ORNs from ribonucleoside 3′-*O*-oxazaphospholidine monomers [80]

synthesized with 97% and 99% coupling efficiency, respectively, at each step (the subscript "S" denotes a phosphorothioate linkage). After removal of the protecting groups, **4** and **5** were isolated by reverse-phase high-performance liquid chromatography (HPLC) in 6% and 11% yield, respectively, and identified by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry [83].

5.2.2 Nuclease Resistance

The resistance of all-(*R*p)-$[U_S]_9U$ (**4**) and all-(*S*p)-$[U_S]_9U$ (**5**) to the exonuclease svPDE [84, 85] and the endonuclease nuclease P1 (nP1) [86] was investigated. These enzymes have often been used for the configurational assignment of PS-ODNs, whereas only a limited number of studies have been reported for stereodefined PS-ORNs. svPDE is *R*p-specific at least for dinucleoside phosphorothioates [76, 87] and oligoribonucleotides containing phosphorothioate linkages [78], whereas nP1 is not always completely *S*p-specific and also hydrolyzes *R*p-phosphorothioate linkages, albeit at a much lower rate than *S*p-phosphorothioate linkages, depending on the nucleobases [75] and lengths [88] of the oligoribonucleotides. In nuclease-resistance experiments [83], all-(*R*p)-$[U_S]_9U$ (**4**) was completely hydrolyzed by svPDE but only 53% hydrolyzed by nP1.



Scheme 4 Synthetic cycle for stereodefined PS-ORNs [80]

Table 4 Digestion of stereodefined PS-ORNs by svPDE and nP1

	Intact PS-ORN remaining (%)	
PS-ORN	svPDE	nP1
All-(*R*p)-[U_S]$_9$U (**4**)	0	47
All-(*S*p)-[U_S]$_9$U (**5**)	77	0

Compounds **4** and **5** were incubated with svPDE (0.1 U) or nP1 (1 U) in a total volume of 20 μL for 16 h and samples analyzed by reverse-phase HPLC

Conversely, all-(*S*p)-[U_S]$_9$U (**5**) was completely hydrolyzed by nP1 but only 23% hydrolyzed by svPDE (Table 4).

5.2.3 Thermal Stability

The effects of the configuration at phosphorus on duplex stability were investigated. Thermal denaturation experiments were conducted on the duplexes of all-(*R*p)-[U_S]$_9$U (**4**), all-(*S*p)-[U_S]$_9$U (**5**), stereorandom [U_S]$_9$U (**6**), and natural [U_O]$_9$U (**7**)

Table 5 Thermal stability of PS-ORNs

Duplex	T_m (°C)
All-(*R*p)-$[U_S]_9U$ (**4**):$[A_O]_9A$ (**8**)	28.9
All-(*S*p)-$[U_S]_9U$ (**5**):$[A_O]_9A$ (**8**)	ND
Stereorandom $[U_S]_9U$ (**6**):$[A_O]_9A$ (**8**)	10.3
$[U_O]_9U$ (**7**):$[A_O]_9A$ (**8**)	25.7

ND melting transition not detected

with the complementary natural $[A_O]_9A$ (**8**) (the subscript "O" denotes a phosphodiester linkage). The T_m value of **4**:**8**, 28.9 °C, was about 3 °C higher than that of its natural counterpart **7**:**8** (Table 5). In contrast, no distinct melting transition was observed above 4 °C for the duplex of **5** with **8**. In addition, a duplex of stereorandom **6** with **8** had a T_m value of only 10.3 °C. Thus, the resulting melting curves show that the thermal stability of the duplexes was appreciably affected by the configuration at phosphorus. Specifically, a backbone consisting of *R*p-phosphorothioate linkages slightly stabilized the PS-ORN–oligoribonucleotide duplex, whereas a backbone consisting of *S*p-phosphorothioate linkages or stereorandom phosphorothioate linkages had a great destabilizing effect on the duplex.

This is consistent with the finding of Yano et al. [79] (see Sect. 5.1) that RNA oligomers with an all-(*R*p) phosphodiester–phosphorothioate alternating backbone synthesized from dimer building blocks have higher affinity for the complementary oligoribonucleotide, while the corresponding all-(*S*p) oligoribonucleotides have similar or lower affinity, than the corresponding unmodified oligoribonucleotide.

5.2.4 Summary

All-(*R*p) oligomer was completely digested by svPDE and partially digested by nP1; the converse was true for the all-(*S*p) oligomer. The complex of all-(*R*p) oligomer with the complementary natural oligomer was slightly stabilized, and the complex of all-(*S*p) oligomer greatly destabilized, relative to the corresponding all-natural complexes.

Studies of diastereomerically pure phosphorothioates oligoribonucleotides synthesized as described in Sects. 5.1.1 and 5.2.1 show that the configuration at the phosphorus atom affects both their nuclease resistance and their duplex-forming ability, so that stereodefined phosphorothioate oligonucleotides of the appropriate configuration should be superior to diastereomixtures. Furthermore, in drug development, diastereomerically pure phosphorothioate oligonucleotides, being single chemical entities, are preferable from the point of view of the specifications of the pharmaceutical product. Stereodefined phosphorothioates synthesized by the above and other methods are expected to find application to nucleic acid drugs, including antisense drugs.

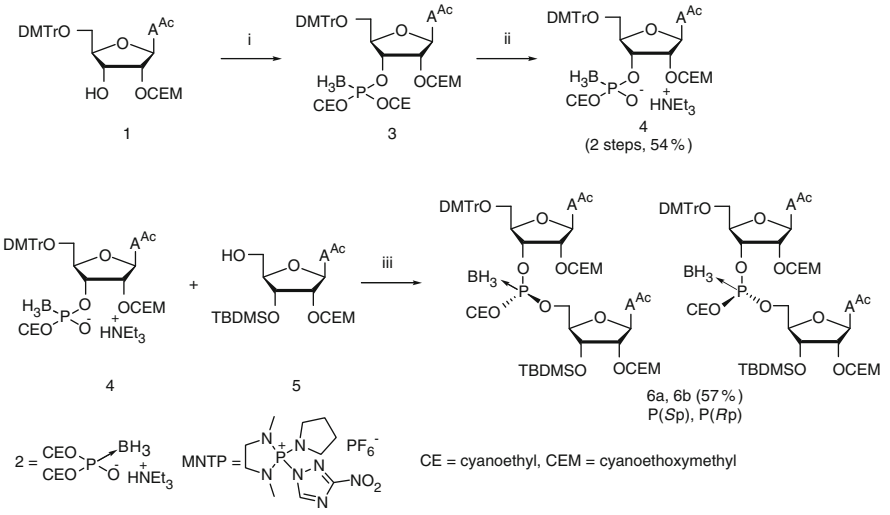
5.3 *Boranophosphates*

5.3.1 Synthesis

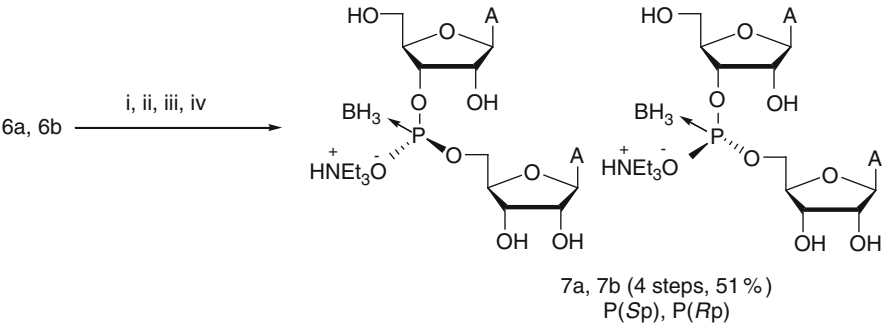
The general properties of boranophosphates were briefly described in Sect. 2.1.3. Since boranophosphate oligodeoxynucleotides (BH_3-ODNs) were first synthesized in 1990 [89], further methods for their synthesis, including both chemical and enzymatic methods, have been reported [90]. However, the enzymes that have been used for the synthesis of BH_3-ODNs, such as T7 RNA polymerase, recognize only the *R*p diastereomer of nucleoside 5′-(α-*P*-borano)triphosphates to form *S*p-boranophosphate internucleotide linkages, so that *R*p internucleotide linkages cannot be generated by these enzymatic methods. For the comparative study of *S*p and *R*p oligomers, therefore, chemical synthetic methods are required. However, there are few reports of the chemical synthesis of boranophosphate oligo*ribo*nucleotides (BH_3-ORNs). In addition, because of undesirable reductions that occur at the *N*-acyl protecting groups of the base moiety during the boronation step, most of the chemical methods reported for the synthesis of BH_3-ODNs and BH_3-ORNs are limited to thymidine and uridine. Although Wada et al. [91] have reported a boranophosphotriester method that can be used to synthesize BH_3-ODNs containing all four nucleobases, there have until recently been no reports of the chemical synthesis of BH_3-ORNs with nucleobases other than uridine.

We have synthesized diastereomerically pure diadenosine 3′,5′-boranophosphates (Ap^bA) by the boranophosphotriester method from ribonucleosides protected with our new 2′-hydroxyl protecting group CEM (2-cyanoethoxymethyl; Scheme 5; see Sect. 6.1) [92]. After synthesis, the other protecting groups of **6a** and **6b** were removed by the procedure devised for 2′-*O*-CEM-protected RNA oligomers to yield the diastereomeric diadenosine boranophosphates **7a** and **7b** (Scheme 6; see Sect. 6.1.2). The two Ap^bA diastereomers were separated by reverse-phase column chromatography for further study. On reverse-phase HPLC, **7a** eluted before **7b** (Fig. 5).

We next investigated the configuration at the chiral phosphorus center in **7a** and **7b** by nuclear magnetic resonance (NMR). The borane groups of the two Ap^bA diastereomers are in different positions relative to the base-stacking region. In the *S*p diastereomer, the borane group is directed toward the base-stacking region and so is close to the H3′ proton of the 5′-residue (pseudoaxial position), while in the *R*p diastereomer, the borane group is directed away from the base-stacking region and so is far from the H3′ proton (pseudoequatorial position). A one-dimensional NOE (nuclear Overhauser effect) difference experiment was carried out to estimate the distance between the BH_3 protons and the H3′ proton of the 5′-residue of each diastereomer. Irradiation of the H3′ proton of the 5′-residue of **7a** resulted in a small increase in the intensity of the BH_3 proton resonances. Similar experiments with **7b** resulted in no change in signal intensity. Although the observed NOE was not large enough to allow definite assignment of the absolute configuration, the results suggest that **7a** is the *S*p diastereomer and **7b** the *R*p diastereomer.



Scheme 5 Synthesis of diadenosine 3′,5′-boranophosphate (**6a**, **6b**). Reagents and conditions: (*i*) **2** (1.2 equiv), 1,3-dimethyl-2-(3-nitro-1,2,4-triazol-1-yl)-2-pyrrolidin-1-yl-1,3,2-diazaphospholidinium hexafluorophosphate (MNTP; 2.4 equiv), and 2,6-lutidine (10 equiv) in CH_3CN, room temperature, 1 h; (*ii*) Et_3N (10 equiv) in CH_2Cl_2, room temperature, 1 h; (*iii*) MNTP (3.0 equiv) and 2,6-lutidine (10 equiv) in CH_3CN, room temperature, 1 h [92]



Scheme 6 Deprotection of diadenosine 3′,5′-boranophosphate (**7a**, **7b**). Reagents and conditions: (*i*) 3% dichloroacetic acid in Et_3SiH/CH_2Cl_2 (1:1, v/v), room temperature, 1 min; (*ii*) Et_3N/CH_3CN (1:1, v/v), room temperature, 1 h; (*iii*) concentrated NH_3 aqueous /EtOH (3:1, v/v), room temperature, 1 h; (*iv*) 1 M tetrabutylammonium fluoride in tetrahydrofuran containing 1% nitromethane, room temperature, overnight [92]

5.3.2 Nuclease Resistance

We investigated the nuclease resistance of the purified Ap^bA diastereomers, which is important not only from a pharmacokinetic but also from a chemical point of view because the results can be used to determine the configuration at phosphorus.

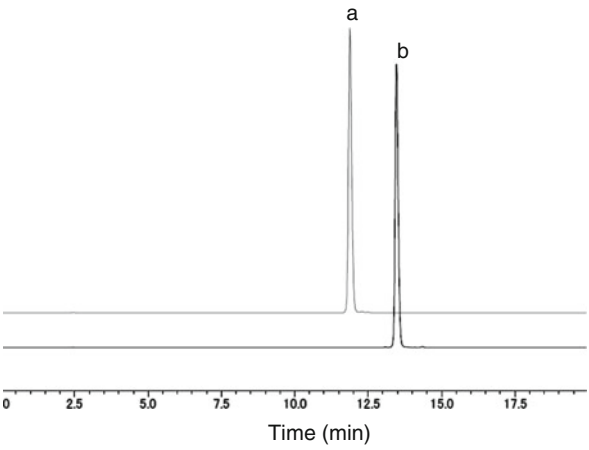
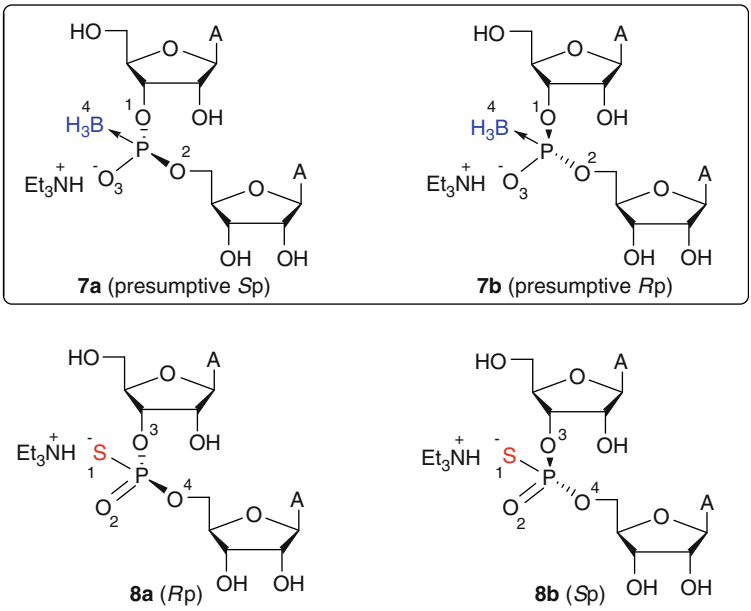


Fig. 5 Reverse-phase HPLC of diadenosine 3′,5′-boranophosphate diastereomers (*a*) **7a** and (*b*) **7b**. Buffer A was 5% CH_3CN, 50 mM triethylammonium acetate, pH 7.0, and buffer B was 90% CH_3CN, 50 mM triethylammonium acetate, pH 7.0. A gradient of 0% to 20% buffer B in 20 min was run at a flow rate of 0.5 mL/min. The column was operated at 40 °C and UV detection was at 260 nm [92]

There is an enzymatic method for determining the configuration at the phosphorus center of phosphorothioates based on the observation that hydrolysis of the *R*p and *S*p diastereomers catalyzed by svPDE proceed at different rates [87]. By the Cahn–Ingold–Prelog priority rules, boranophosphate and phosphorothioate linkages with the same configuration in space have opposite *R* and *S* designations (Scheme 7). The boranophosphate linkage that is resistant to svPDE has been proposed to have the *R*p configuration [93, 94]. Boranophosphate dimers have higher resistance to svPDE than natural dimers do, and the *R*p diastereomer, which elutes more slowly on reverse-phase HPLC, is more resistant than the *S*p diastereomer.

To investigate the effect of modifications at phosphorus on nuclease resistance, we compared the resistance to svPDE and nP1 of the five dimers ApA, Ap^bA (faster-eluting; **7a**), Ap^bA (slower-eluting; **7b**), Ap^sA (*R*p; **8a**), and Ap^sA (*S*p; **8b**) by incubation for 24 h with either enzyme (Table 6). The boranophosphate dimers **7a** and **7b** and the phosphorothioate dimers **8a** and **8b** were significantly more resistant to both enzymes than was the natural dimer ApA, which was completely degraded by both enzymes within 30 min. In particular, **7b** and **8b** were more resistant to svPDE than the other dimers were. From this diastereospecific resistance to svPDE, we infer that **7b** corresponds to the *R*p configuration, an inference that is consistent with the NMR measurements and that provides independent support for the assignment of configuration. Boronation was as effective as sulfurization in providing resistance to svPDE. Compounds **7b** and **8a** were equally resistant to nP1. With respect to the *R*/*S* designations, the rank order of resistance to nP1 observed for the boranophosphate diastereomers, **7a** (presumptive *S*p) < **7b** (presumptive *R*p), was the opposite of that observed for the phosphorothioate diastereomers, **8b** (*S*p) < **8a** (*R*p). With respect to the absolute stereochemical specificity of the enzyme, however, the rank order of resistance to nP1 was, as might be expected, the same for both chemical modifications.



Scheme 7 Absolute structure of boranophosphate and phosphorothioate diastereomers and their *R*/*S* designations. The substituents at the phosphorus atom are numbered from *1* (highest priority) to *4* (lowest priority) in decreasing order of the atomic number of the atoms attached to the stereogenic center according to the Cahn–Ingold–Prelog priority rules. Note that boranophosphate and phosphorothioate diastereomers with the same absolute configuration in space have opposite *R*/*S* designations [92]

Table 6 Digestion of dimers by snake venom phosphodiesterase or nuclease P1

	Intact dinucleotide remaining (%)	
Dimer	svPDE	nP1
ApA (natural)	0	0
Ap^bA (*S*p; **7a**)	10	0.4
Ap^bA (*R*p; **7b**)	83	77
Ap^sA (*R*p; **8a**)	2.8	75
Ap^sA (*S*p; **8b**)	96	0

Each dimer was incubated with svPDE (0.3 U) or nP1 (2 U) for 24 h and samples were analyzed by reverse-phase HPLC

5.3.3 Thermal Stability

The adenosine dimer ApA forms an ApA:2poly(U) triple-helical complex in the presence of 1 M NaCl [95, 96]. We performed mixing experiments to investigate the stoichiometry of the interaction between Ap^bA and poly(U). The mixing curves for Ap^bA **7a** or **7b** and poly(U) in the presence of 1.0 M NaCl revealed a break at Ap^bA:poly(U) = 1:2, indicating the formation of an Ap^bA:2poly(U) triple-helical complex. We then measured the T_m values of the dimer:2poly(U) triple-helical

Table 7 Melting temperatures of dimer:2poly (U) complexes and dimer:2poly(dT) complexes

	T_m (°C)	
Dimer	With poly(U)	With poly(dT)
ApA	9.1	−1.9
Ap^bA (*Sp*; **7a**)	16.0	−2.8
Ap^bA (*Rp*; **7b**)	4.0	−0.5

complexes to compare the hybridization properties of ApA, Ap^bA (**7a**) and Ap^bA (**7b**) in the presence of 1.0 M NaCl (Table 7). The T_m value of the **7a**:2poly(U) complex (16 °C) was substantially higher than that of the ApA:2poly(U) complex (9.1 °C). In contrast, the T_m value of the **7b**:2poly(U) complex (4.0 °C) was substantially lower than that of ApA:2poly(U) complex. Dimer **7a** is proposed to have the *S*p configuration on the basis of NMR measurements and its susceptibility to hydrolysis by svPDE. These results indicate that the *S*p Ap^bA dimer hybridizes more strongly to poly(U) than the natural dimer does. The T_m values of the dimer:2poly(dT) complexes were similar, and below 0 °C (Table 7). Therefore, the increase in the strength of hybridization between *S*p Ap^bA dimer and polymer is specific for the RNA polymer. These findings point the way to the development of a nucleic acid to recognize RNA with higher specificity than natural oligomers do.

5.3.4 Summary

These preliminary studies on the physicochemical properties of diastereomerically pure oligoribonucleotide boranophosphate dimers suggest that BH_3-ORNs synthesized with the *S*p configuration can be developed as a promising new kind of RNA that will from stable complexes with complementary RNA. Boranophosphate RNA has the advantages of chemical and biological stability, high ability to cross the cell membrane, low toxicity, applicability to boron neutron capture therapy, and high RNAi activity. These make it a good candidate for chemically modified antisense RNA diagnostics and therapeutics.

6 Chemical Synthesis of Long RNA Oligomers

Cost-effective synthesis of both long and short RNA is needed for scaled-up production for therapeutic and diagnostic applications. The usual methods for preparing long RNA oligomers are in vitro transcription with T7 RNA polymerase or the use of an expression vector. However, these methods do not give sufficiently pure long RNA oligomers, nor can they be easily scaled up for production. In addition, various errors and inefficiencies are associated with in vitro transcription [97]. Therefore, a chemical method of RNA synthesis that is not only efficient and cost-effective but also that could be extended to the production of long RNA oligomers is highly desirable.

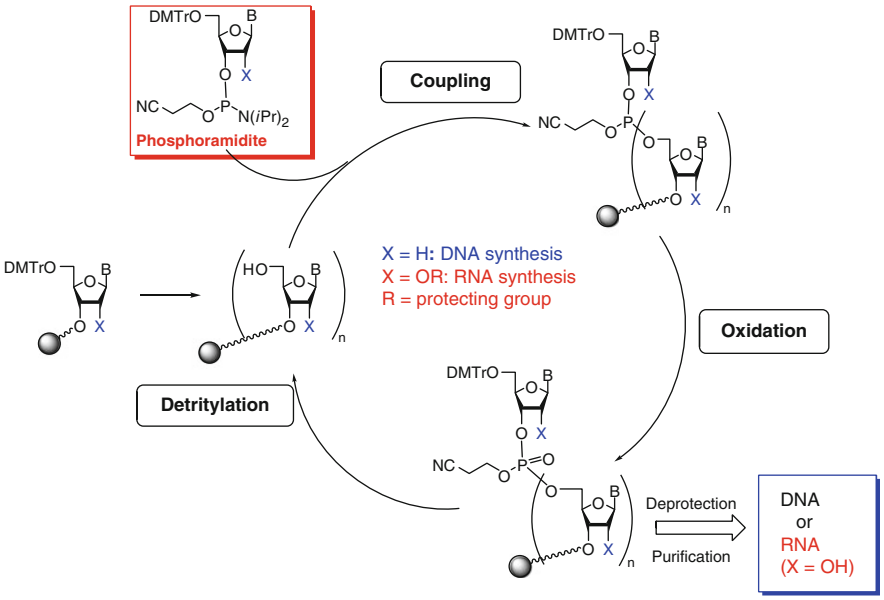
Because of the need to protect the 2′-hydroxyl group of the ribose moiety during the synthetic cycle, RNA is more difficult to synthesize than DNA, and the success of an RNA synthetic method largely depends on the selection of a suitable 2′-hydroxyl protecting group. We have developed a new solid-phase method for the synthesis of RNA oligomers with CEM as the 2′-hydroxyl protecting group. To verify the potential of the method, over a period of several years we synthesized a series of RNA oligomers of increasing length, including shRNA, pre-miRNA, and designed mRNA sequences up to 170 nt long, and confirmed the identity of the oligomers by physicochemical and biological methods. In this section we describe the CEM method, emphasizing the following advantages of CEM as a 2′-hydroxyl protecting group: (1) its ease of introduction at the 2′ position, (2) its small molecular size, which minimizes steric hindrance during chain elongation, and (3) its ease of removal under mild conditions after oligomer synthesis. The CEM method is suitable for application to the synthesis of very long RNA oligomers and is expected to be particularly advantageous for incorporating chemically modified nucleotides into long RNA oligomers.

6.1 The CEM Method

A general procedure for the solid-phase synthesis of oligonucleotides by the phosphoramidite method is shown in Scheme 8. The synthetic cycle consists of three major stages: coupling, oxidation, and detritylation.

For the 5′-hydroxyl function, the most widely used protecting group is the 4,4′-dimethoxytrityl (DMTr) group. With DMTr as the 5′-hydroxyl protecting group, the 2′-hydroxyl function is usually protected with a fluoride-cleavable silyl group [99], a photocleavable group [100], or an acid-cleavable acetal [101, 102]. TBDMS [99] is a popular choice of 2′-hydroxyl protecting group, and the protected phosphoramidites (amidites) are commercially available. Nevertheless, primarily because of its large steric bulk and relative difficulty of removal after oligomer synthesis, TBDMS as a 2′-hydroxyl protecting group leaves room for improvement in the yield and purity of the final product [103–106]. In particular, the synthesis of RNA oligomers longer than about 50 nt by this method is extremely difficult. To solve these problems, several new 2′-hydroxyl protecting groups with smaller steric bulk than TBDMS, such as bis(2-acetoxyethoxy)methyl (ACE) [107] and triisopropylsilyloxymethyl (TOM) [108], have been developed (Fig. 6).

Pfleiderer and coworkers [102] investigated the use of acetal groups with electron-withdrawing substituents, such as 2-cyanoethoxyethyl (CEE), as 2′-hydroxyl protecting groups (Fig. 6). However, such protecting groups were found to be too acid-stable to be removed under normal deprotection conditions, and their development as acid-cleavable 2′-hydroxyl protecting groups was abandoned. These workers observed unwanted loss of the CEE group in polar aprotic



Scheme 8 Solid-phase synthesis of DNA or RNA [98]

solvents due to fluoride anion, but this loss could be prevented by adding acetic acid. Wada and coworkers [109] later took advantage of this selective fluoride cleavability of CEE in aprotic solvents to demonstrate the usefulness of CEE as a 2′-hydroxyl protecting group in the synthesis of dinucleotides.

With these considerations in mind, we developed a novel solid-phase RNA synthetic method based on the use of CEM as the 2′-hydroxyl protecting group [98, 110–112]. The CEM method has the following advantages:

1. Ease of preparation of 2-cyanoethyl chloromethyl ether (CEM-Cl) and 2-cyanoethyl methylthiomethyl ether, novel alkylating agents for introducing CEM at the 2′ position, from moderately priced starting materials
2. Ease of introduction of the CEM group at the 2′ position, which facilitates the large-scale production of nucleotide monomer block
3. The small molecular size of the CEM group, which creates correspondingly low steric hindrance at the elongation reaction site and therefore allows a high coupling yield in oligomer synthesis
4. A minimum number of substituents on the carbon attached to the 2′ oxygen (ribose-2′-O–CH_2–O–R) of the CEM group, so that no stereogenic center is generated during the attachment of the protecting group
5. Ease of analysis of the CEM-protected RNA by HPLC for monitoring the deprotection steps

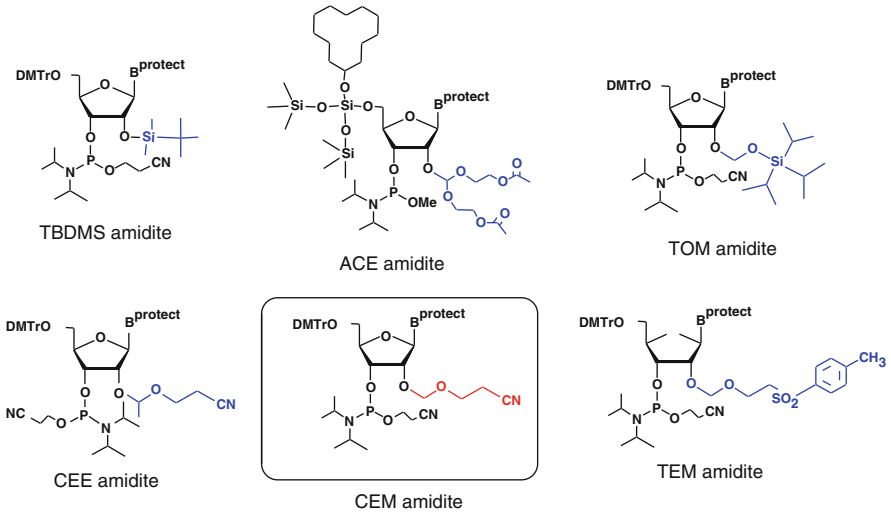


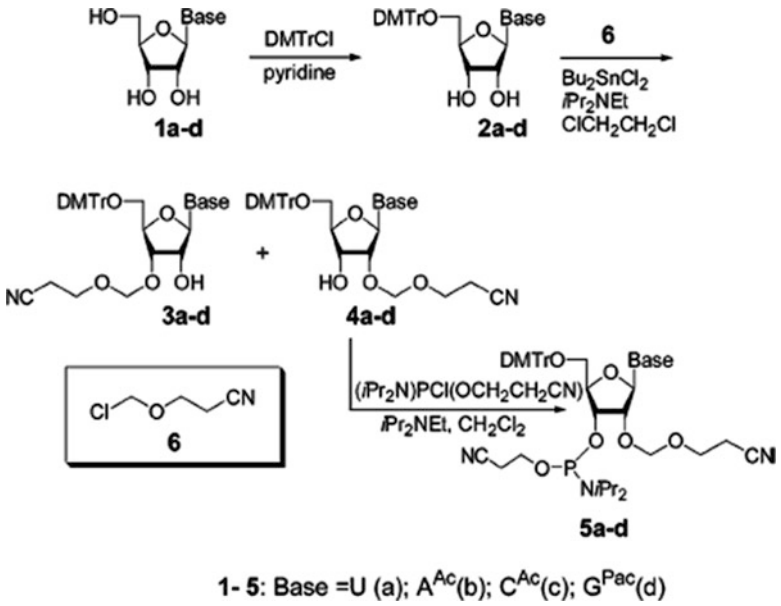
Fig. 6 2′-Hydroxyl protecting groups for RNA synthesis [98]

6. Rapid and complete post-synthetic removal of the protecting group by fluoride anion (for oligomers of <25 nt, removal is complete after 1 h at room temperature, compared to 8 h at room temperature for the TBDMS group)
7. Compatibility of the deprotection procedure with standard, unmodified DNA synthesizer equipment

After our discovery of CEM as a new 2′-hydroxyl protecting group, Chattopadhyaya and coworkers [113] reported the use of 2-(4-tolylsulfonyl) ethoxymethyl (TEM) as an alternative 2′-hydroxyl protecting group based on CEM. Although TEM is slightly more stable than CEM under basic conditions, it is somewhat more difficult to remove completely after synthesis, so that it may be difficult to apply it to the synthesis of very long RNA oligomers.

6.1.1 CEM Phosphoramidites

We will now describe the synthesis of the CEM amidites and, in subsequent sections, give examples of their use in the solid-phase synthesis of RNA oligomers. The phosphoramidites **5a–d** were synthesized according to Scheme 9. Starting with a suitable base-protected nucleoside, **1a–d**, we derivatized the 5′-hydroxyl group with DMTr and then the 2′-hydroxyl group with CEM. The latter derivatization was carried out via the 2′,3′-*O*-dibutylstannylidene intermediate, which was treated with CEM-Cl (**6**; 2-cyanoethyl chloromethyl ether) to give a mixture of the 3′-*O*-CEM and 2′-*O*-CEM derivatives (**3a–d** and **4a–d**). After isolating the 2′-*O*-CEM derivatives **4a–d** by silica gel column chromatography, we carried out phosphitylation of the



Scheme 9 Preparation of phosphoramidites [110]

3′-hydroxyl group to obtain the corresponding amidites **5a–d**. CEM-Cl itself was prepared via the Pummerer reaction. Briefly, 3-hydroxypropionitrile and dimethyl sulfoxide were reacted to give the methylthiomethyl ether, which was then treated with sulfuryl chloride to give CEM-Cl.

6.1.2 Synthesis of a Homo-Oligomer

As a first test of CEM chemistry, we synthesized a uridine homo-oligomer, U_{40} [110]. Commercially available controlled-pore glass derivatized with 2′- or 3′-*O*-benzoyl-rU was used as the solid support and 5-ethylthiotetrazole as the activator. Cleavage from the resin and deprotection of the phosphate moiety were carried out by treatment with concentrated ammonia in EtOH at 40 °C for 4 h. At this stage, the relative hydrophilicity of the 2′-*O*-CEM group allows monitoring of the 2′-*O*-CEM-protected U_{40} by HPLC (Fig. 7a). In the next step, the 2′-*O*-CEM protecting group was removed by treatment with 1 M tetrabutylammonium fluoride/tetrahydrofuran for several hours. HPLC profiles are shown for the fully deprotected crude U_{40} prepared by the CEM method (Fig. 7b) and deprotected DNA of the same chain length (dT_{40}; Fig. 7c). Comparison of the HPLC profiles of the entire crude reaction mixtures shows that the CEM method gave RNA of more than 80% purity, similar to the purity obtained at the same stage of DNA synthesis.

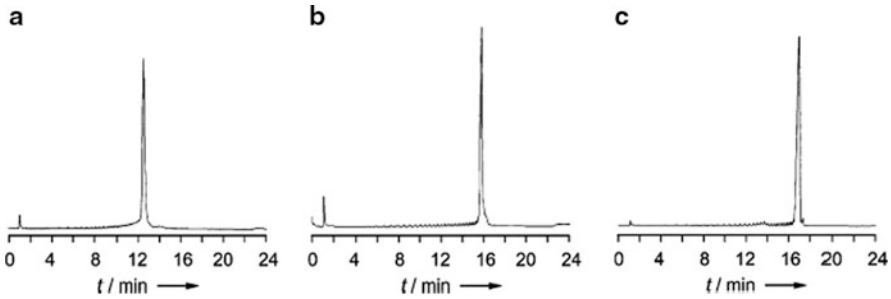


Fig. 7 Anion-exchange HPLC of homo-RNA and DNA. (**a**) Unpurified 2′-*O*-CEM-protected U_{40}. Buffer A, 10% CH_3CN, 25 mM Tris-HCl, pH 8.0; buffer B, 10% CH_3CN, 25 mM Tris-HCl, pH 8.0, containing 700 mM $NaClO_4$; gradient from 5% to 50% buffer B in 20 min; flow rate, 1.5 mL/min; 50 °C. (**b**) Unpurified fully deprotected U_{40}. Gradient from 5% to 50% buffer B in 20 min. (**c**) Unpurified fully deprotected dT_{40} (DNA). Buffer C, 25 mM Tris-HCl, pH 8.0; Buffer D, 25 mM Tris-HCl, pH 8.0, containing 700 mM $NaClO_4$; gradient from 10% to 30% Buffer D in 20 min; flow rate, 1 mL/min; 40 °C. UV detection was at 260 nm [110]

6.2 Pre-miRNA

6.2.1 Synthesis

miRNAs are transcribed as long primary miRNAs that undergo processing through a pre-miRNA intermediate to the mature miRNA duplex [114]. As a test of the suitability of CEM chemistry for the synthesis of long RNA oligomers that would be more rigorous than the synthesis of shRNA (see Sect. 6.2.2), we set out to synthesize a 110mer with the sequence of the candidate pre-miRNA, pre-miR-196a-2 (Fig. 8) [111]. After preparative reverse-phase HPLC, the highly pure 110mer was obtained in a good overall yield of 5.5%.

6.2.2 Physicochemical Identification

The purity of the final product was confirmed by anion-exchange HPLC (Fig. 9). Polyacrylamide gel electrophoretic analysis (Fig. 10) was consistent with the synthesis of a 110mer, and the structure of the product was confirmed by mass spectrometry after digestion by the sequence-specific endoribonuclease MazF [115].

6.2.3 mRNA Expression Analysis

To assess the biological activity of the chemically synthesized 110-nt pre-miRNA candidate, we measured its specific gene-silencing effect by two methods, mRNA-

Fig. 8 Pre-miRNA candidate pre-miR-196a-2. The *grey* part indicates the sequence of miRNA-196-a [111]

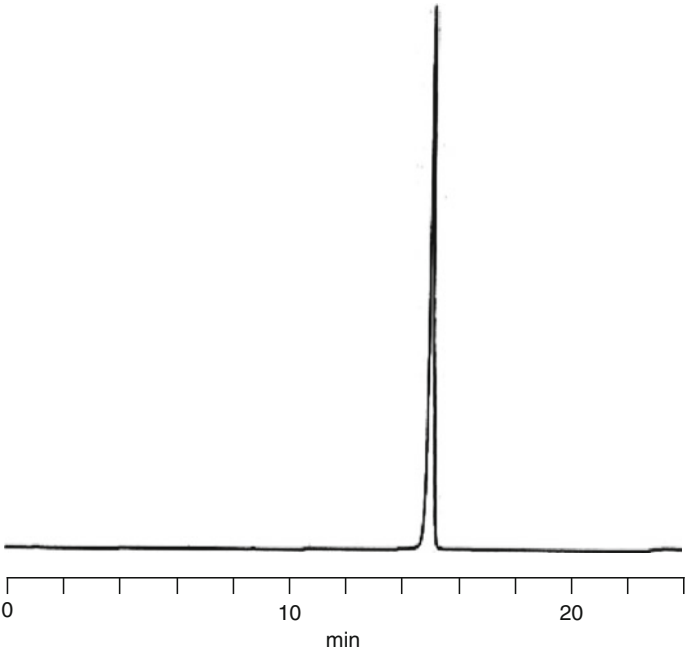


Fig. 9 Anion-exchange HPLC of purified 110-nt RNA. The solvent system was buffers A and B, which are described in the legend to Fig. 7. Elution was performed with a gradient from 5% to 50% buffer B in 20 min [111]

expression analysis by real-time polymerase chain reaction (RT-PCR) and luciferase reporter assay. miR-196a has complementarity to sites in the 3′-UTR of homeobox (HOX) genes, including the human *HOXB8*, in which the pairing is perfect except for a single mismatch, and *HOXC8*, in which the pairing is less perfect. HOX gene expression has been suggested to be post-transcriptionally regulated by miR-196a [116]. To test whether the chemically synthesized pre-miRNA had silencing activity against the endogenous target gene, we assessed its inhibition of endogenous *HOXC8* mRNA expression in PC-3 cells, a human prostate cancer cell line, by RT-PCR. For comparison, we also assessed the inhibition of mRNA expression by the 22-nt miR-196a RNA duplex. The chemically synthesized pre-miRNA was found to decrease target mRNA levels as effectively as the 22-nt miR-196a RNA duplex (Fig. 11).

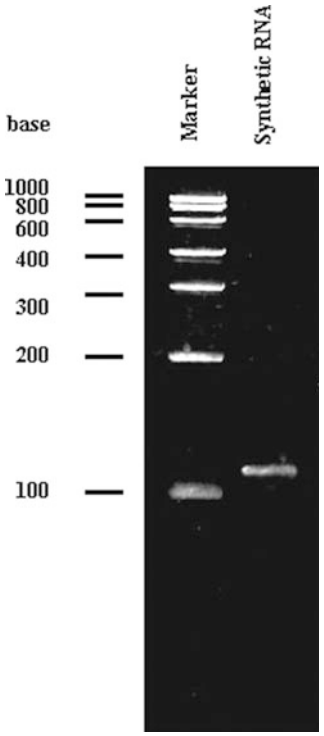


Fig. 10 Electrophoresis of purified 110-nt RNA. The synthetic RNA was analyzed on a 5% polyacrylamide gel and stained with the cyanine dye SYBR Green II [111]

6.2.4 Luciferase Reporter Assay

To test the gene-silencing effect of the chemically synthesized 110-nt pre-miRNA by an independent method, we used a reporter assay system with a reporter gene whose expression can be regulated by miR-196a. DNA duplex corresponding to the *HOXB8* target sequence and its flanking region was inserted into a luciferase-expressing reporter plasmid. In this system, silencing by miR-196a is observed as a reduction in luciferase expression. Transfection with the synthetic pre-miRNA decreased the expression of the target gene about as effectively as the 22-nt miR-196a RNA duplex (Fig. 12, left). The suppression of luciferase activity by the synthetic pre-miRNA could have resulted from the inhibition of luciferase expression at either the transcriptional or the translational level.

Although the guide (antisense) strand of an siRNA molecule is normally specifically incorporated into the RNA-induced silencing complex (RISC), it sometimes happens that the passenger (sense) strand is incorporated instead, producing off-target effects [118, 119]. A pre-miRNA molecule would be expected to interact more specifically with RISC to exclude the sense strand during processing of the pre-miRNA, resulting in more-specific cleavage of the target mRNA [118–120].

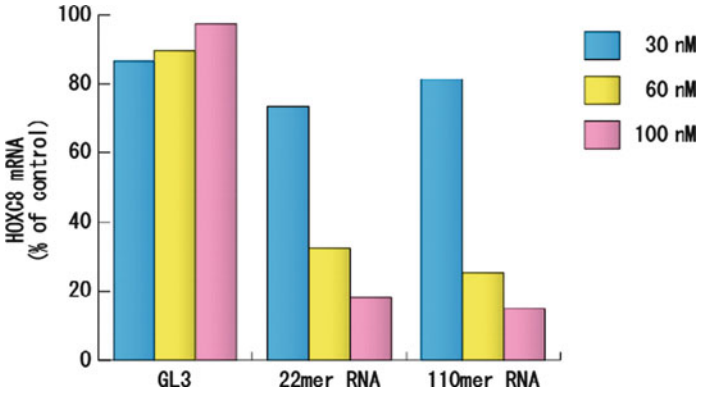


Fig. 11 *HOXC8* mRNA expression analysis by RT-PCR PC3 cells expressing HOXC8 were transfected with the 110-nt pre-miRNA or the 22-nt miRNA duplex and *HOXC8* mRNA was determined by RT-PCR after 24 h. *HOXC8* mRNA expression was normalized to that of glyceraldehyde-3-phosphate dehydrogenase [117]

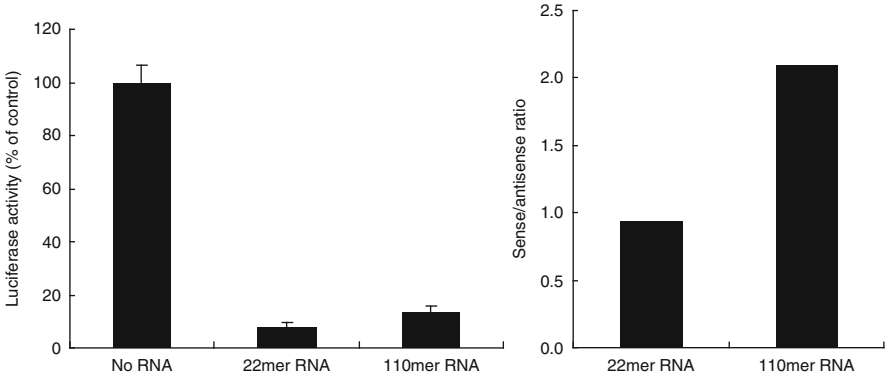


Fig. 12 Gene-silencing effect of chemically synthesized pre-miRNA. *Left*: Effect of pre-miRNA on expression of luciferase target gene. G3T-hi cells were transfected with pHOXB-Luc reporter plasmid, and effector RNA (100 nM) and reporter assays performed 48 h after transfection. Each value shown is the average of three independent experiments, and standard deviations are indicated as error bars. *Right*: Sense/antisense suppression activity ratios. G3T-hi cells were transfected with pHOXB-Luc or pHOXB-Luc-antisense reporter plasmid and effector RNA (30 nM), and reporter assays performed 48 h after transfection. The results are presented as the ratio of the average percent suppression of luciferase-containing sense and antisense target by the 110-nt pre-miRNA or the 22-nt miRNA duplex [111]

The silencing effect of pre-miR-196a against the sense target was about twice as strong as that against the antisense target, whereas the 22-nt miR-196a duplex showed about the same silencing effect against both targets (Fig. 12, right). The observation of an equal silencing effect against the sense and antisense target mRNA by the 22-nt duplex could be due to a comparable stability of the sequences at the ends of the duplex, which might prevent preferential incorporation of

the guide strand [121, 122]. The 110-nt pre-miRNA, therefore, showed a strand selectivity in gene silencing that was not shown by the miRNA.

6.2.5 Summary

We have prepared a 110-nt pre-miRNA by chemical synthesis in a single synthesizer run, confirmed its structure by physicochemical methods, including mass spectrometry, and shown that it had biological activity. Because pre-miRNA has to undergo processing before it can exert its gene-silencing effect, we conclude that the synthetic 110-nt pre-miRNA was processed within the cell and that the resulting miRNA was able to mediate gene silencing. This work demonstrates the suitability of the CEM method for the synthesis of biologically active RNA oligonucleotides up to 110 nt in length.

6.3 Single and Double Short Hairpin RNA

As mentioned in the Introduction and described in Sect. 3, therapies based on siRNA-mediated gene silencing are under development. As an alternative to introducing a gene-targeting siRNA into the cell, shRNA [123] can be introduced into the cell instead. shRNA is a single RNA strand 50–60 nt in length with a tight hairpin turn, an intramolecular double-stranded region, and a 2-nt 3′ overhang. In cells, it is cleaved by Dicer into siRNA. Gene silencing mediated by shRNA is at least as effective as that mediated by siRNA or miRNA targeting the same sequence [124].

The production of siRNA requires the separate synthesis and purification of two strands, which, after freeze-drying, must be dissolved and annealed. In contrast, shRNA, being a single strand with an intramolecular double-stranded structure, does not require the annealing of separate strands and so is more suitable for industrial production.

shRNA offers a fresh hope for RNAi therapeutic applications, and chemically modified shRNA is under investigation in our and other laboratories [125]. In Sects. 6.3.1–6.3.6, we introduce our recent work on a related molecule, dshRNA, which consists of two identical shRNA molecules covalently linked through their 3′ ends.

6.3.1 Molecular Design

dshRNA is the fruit of our efforts to realize gene-silencing RNA with greater nuclease resistance and silencing activity than siRNA or shRNA, yet with weaker induction of the innate immune response. We wanted these improvements to be, as far as possible, inherent in the molecular design of the RNA rather than the result of

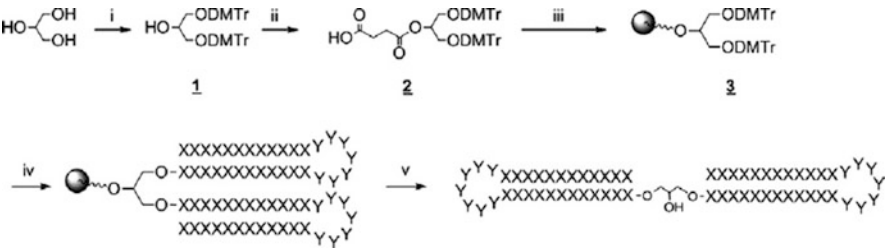


Fig. 13 Strategy for solid-phase synthesis of dshRNA. (*i*) DMTr protection of glycerol. (*ii*) Attachment of succinyl linker to glycerol. (*iii*) Attachment of glycerol to long-chain alkylamine controlled-pore glass solid support through succinyl linker. (*iv*) Oligonucleotide synthesis on DNA/RNA synthesizer. (*v*) Cleavage from solid support and deprotection. *X* nucleotide in duplex, *Y* nucleotide in loop [127]

chemical modification, because excessive modification can diminish the silencing activity [126]. dshRNA is expected to be more resistant to 3′–5′ exonucleases than siRNA or shRNA because there is no free 3′ end for such nucleases to act on.

6.3.2 Synthesis

The CEM method was used to synthesize a series of dshRNA molecules, each of which was synthesized in a single run on a DNA/RNA synthesizer without the need for enzymatic ligation. Synthesis was carried out on a solid support derivatized with glycerol (Fig. 13) and the final products dsh1–dsh5 (Fig. 14) were identified by electrospray ionization mass spectrometry. The dshRNAs were all targeted to the same mRNA sequence corresponding to a part of the Bcl-2 protein [71].

6.3.3 Structure and Gene-Silencing Activity

We tested the gene-silencing activity of the dshRNAs by measuring their suppression of Bcl-2 protein production in A431 cells. The silencing activity of dsh1–dsh5 was measured at concentrations of 3 and 10 nM (Fig. 15), with B717, an siRNA with known Bcl-2-suppressing activity, as the positive control (see Sect. 4).

When the shRNA elements were linked through their sense rather than their antisense sequences, as in dsh3, Bcl-2 was almost completely suppressed at only 3 nM. This activity was even higher than that of the positive-control B717 siRNA, probably because dshRNAs contain twice as many mole equivalents of the antisense sequence per molecule as the siRNA.

6.3.4 Exonuclease Resistance

The resistance of dsh3 to the 3′–5′ exonuclease svPDE was assessed. Before incubation with svPDE, RNA samples were annealed to form the duplex structures,

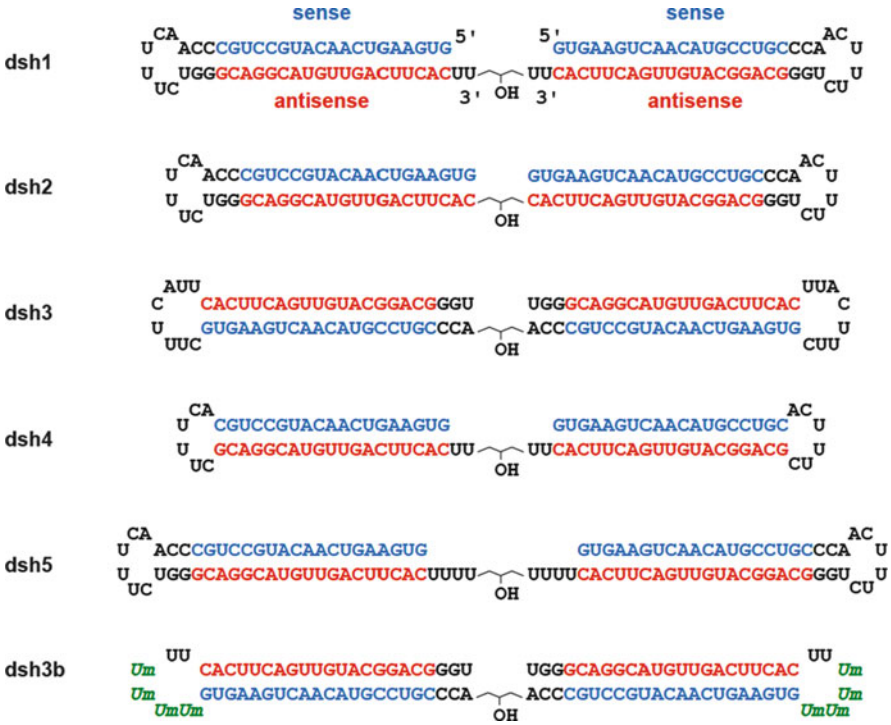


Fig. 14 Sequences and structures of synthetic dshRNAs. *dsh1*: 22-bp duplex, 2-nt single-stranded link between duplex and glycerol, attachment of shRNA to glycerol through antisense region. *dsh2*: 22-bp duplex, attachment to glycerol through antisense region. *dsh3*: 22-bp duplex, attachment to glycerol through sense region. *dsh4*: 19-bp duplex, 2-nt single-stranded link between duplex and glycerol, attachment to glycerol through antisense region. *dsh5*: 22-bp duplex, 4-nt single-stranded link between duplex and glycerol, attachment to glycerol through antisense region. *dsh3b*: 22-bp duplex, attachment to glycerol through sense region, inclusion of 2′-*O*-methyluridine (*Um*) in loop [127]

and after incubation the percentage of intact full-length RNA remaining was determined by HPLC. siRNA was completely digested after incubation for 2 h, while shRNA, with its two-nucleotide 3′ overhang, was digested to the extent of 56% under the same conditions. Product dsh3, however, which is 3′–3′-linked through glycerol, was only digested to the extent of 16%. Thus, the rank order of svPDE resistance was dshRNA > shRNA > siRNA, with dshRNA being the most resistant of all the RNA structures tested.

6.3.5 Induction of Interferon-α

In designing dsh3b with 2′-O-methylated loop residues to suppress the innate immune response, we assumed that, for high gene-silencing activity of the antisense sequences, two natural nucleotides would be needed to form an overhang at the 3′

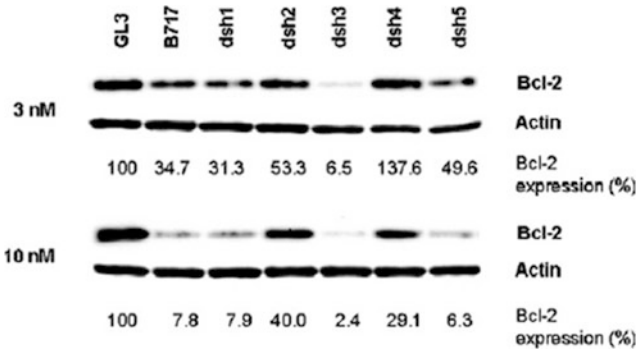


Fig. 15 Suppression of Bcl-2 gene expression by dsh1–dsh5. Western blot analysis of the expression of Bcl-2 protein in A431 cells 72 h after transfection with dshRNA complexed with LIC-101. Actin was used as an internal control. Luciferase GL3 siRNA was used as the negative control and B717 siRNA as the positive control. See the legend to Fig. 2 for the sequences of the sense and antisense strands [127]

end of the 19-nt antisense sequence after enzymatic processing in the cell. Therefore, we designed dsh3b with loops consisting of two natural nucleotides at the 3′ end of the 19-nt antisense sequence and four 2′-*O*-methyluridines. dsh3b was fully active, indicating that activity was not affected by loop size or the nature of the loop components. We compared the ability of dsh3 and dsh3b to induce an innate immune response, as measured by the production of interferon-α (IFN-α) by human peripheral blood mononuclear cells (Fig. 16). dsh3b, which differs from dsh3 only in the loop, showed up to 58% lower induction of IFN-α than siRNA did, with no loss of gene-silencing activity. The lower induction of IFN-α is presumably due to the 2′-*O*-methyluridine residues in the loop. The inclusion of 2′-*O*-methyluridine residues in an siRNA sequence has previously been shown to suppress immune activation [128], but there are no reports of the suppression of cytokine induction by the inclusion of 2′-*O*-methyluridine residues in shRNA. Modification of the loop of shRNA and dshRNA might be a useful general strategy for suppressing their tendency to induce cytokines.

6.3.6 Summary

We have synthesized a series of dshRNAs consisting of two identical shRNA elements covalently linked by glycerol through their 3′ ends. This study provides the first reported example of 3′–3′-linked shRNAs. When the core effector duplex was identical, linking the 3′ ends of the two sense regions with glycerol was associated with especially high gene-silencing activity. dsh3b, which has 2′-*O*-methyluridine residues in the loop, showed lower induction of IFN-α than siRNA did. It also showed higher exonuclease resistance than siRNA while retaining its gene-silencing activity.

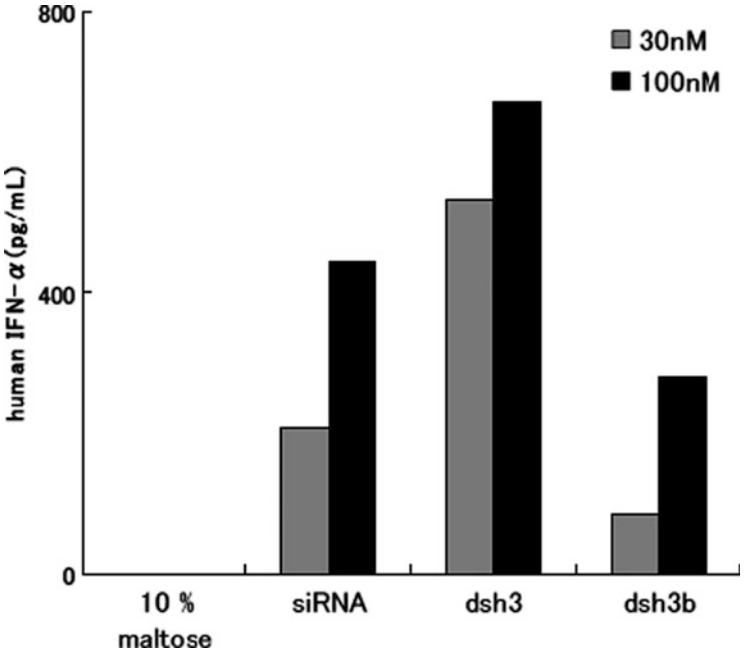


Fig. 16 Induction of IFN-α by dshRNA and siRNA. Human peripheral blood mononuclear cells were treated for 24 h with dshRNA complexed with LIC-101, and IFN-α levels measured by enzyme-linked immunosorbent assay (ELISA). Maltose solution (10% w/v) was used as the negative control and B717 siRNA as the positive control [127]

6.4 Artificial mRNA

In the antisense strategy, mRNA is the sense strand and the target for gene silencing. However, there is also the possibility of developing mRNA itself for medical application by a chemical synthetic approach. In addition, synthetic mRNA would be a useful tool for the intracellular expression of oligopeptides in basic research. In anticipation of future therapeutic and research requirements for mRNA, and as a more stringent test of the CEM method than that described in Sect. 6.2.1, we developed a chemical approach to the synthesis of mRNA.

6.4.1 Molecular Design

We designed a 130-nt mRNA sequence [129] encoding a 33-amino-acid peptide that included the sequence of glucagon-like peptide-1 (GLP-1), a peptide that stimulates glucose-dependent insulin secretion from the pancreas [130]. The 130-nt sequence consisted of a 20-nt 5′-UTR followed by the GLP-1-coding region and a 3′-UTR. To increase the efficiency of translation, the 5′-UTR and the start of the coding region were designed to include a Kozak consensus sequence, which is

characterized by a purine three bases upstream of the start codon and a guanine immediately downstream [131].

6.4.2 Synthetic Strategy

After chemical synthesis of the mRNA sequence followed by pyrophosphorylation at the 5′ end, the preparation of the artificial mRNA was completed by the enzymatic addition of a 5′ cap and a 3′ poly(A) tail. The synthetic strategy (Fig. 17) involved:

1. Solid-phase synthesis of the RNA sequence from CEM amidites
2. Monophosphorylation and pyrophosphorylation of the 5′ end of the RNA while it was still attached to the solid support
3. Cleavage of the pyrophosphorylated RNA from the solid support followed by deprotection and purification
4. Addition of the 5′ cap with capping enzyme
5. Polyadenylation of the 3′ end with poly(A) polymerase

Because existing phosphorylating reagents are insufficiently reactive for the pyrophosphorylation of long RNA oligomers attached to a porous solid support, we developed more-reactive reagents. We also synthesized a 170-nt RNA molecule that contained, in addition to the 130-nt sequence described above, a chemically synthesized 40-nt poly(A) tail instead of the enzymatically added poly(A) tail. The synthetic poly(A) tail has an advantage from the point of view of the specifications of a pharmaceutical product because the mRNA would be a single molecular species, in contrast to the mRNA with a distribution of poly(A) tail lengths obtained with the use of poly(A) polymerase.

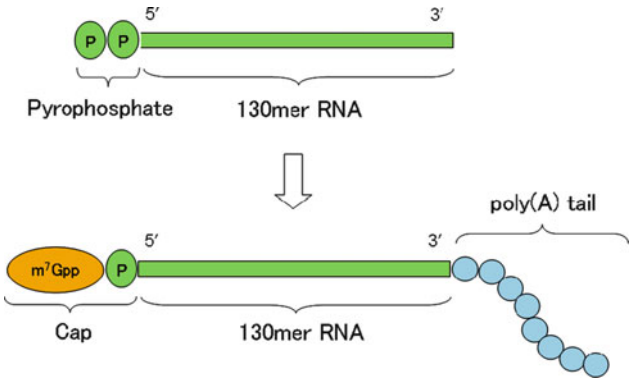


Fig. 17 Enzymatic conversion of 130mer to artificial mRNA [129]

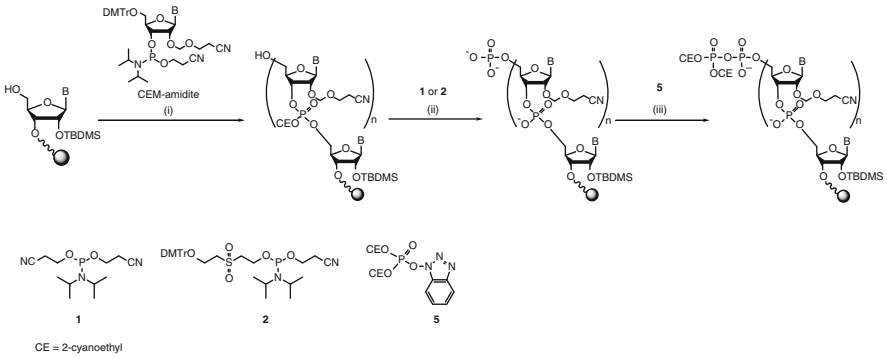
6.4.3 Synthesis, Phosphorylation, and Pyrophosphorylation

The 130-nt RNA oligomer was synthesized by the CEM method as in our previous synthesis of a 110mer (Scheme 10; see Sect. 6.2.1). The 130-nt RNA, while still on the solid support, was monophosphorylated with phosphorylating reagent **1** or **2** (Scheme 10) in almost quantitative yield with either reagent.

For pyrophosphorylation, we developed a series of novel reagents with a phosphotriester structure, which is much more reactive than the phosphodiester structure of existing reagents. One of these, **5**, was found to be especially suitable for the pyrophosphorylation of long RNA and was used for the pyrophosphorylation of the 5′-monophosphorylated mRNA sequences used in this study (Scheme 10). After cleavage from the solid support, deprotection, and purification, the total yields of 130- and 170-nt 5′-pyrophosphorylated oligomers, including both the synthetic and the pyrophosphorylation steps, were 8.0% and 4.8%, respectively, corresponding to respective average coupling yields of 98.1% and 98.2%. Their purity was confirmed by HPLC (Fig. 18).

6.4.4 Capping and Polyadenylation

The cap 1 structure was attached to the 5′ end of the 5′-pyrophosphorylated 130- or 170-nt RNA oligomer by the use of a combination of a capping system and 2′-*O*-methyltransferase. The capping system catalyzes the formation of the cap 0 structure from guanosine triphosphate and 5′-pyrophosphorylated RNA, while 2′-*O*-methyltransferase completes the formation of the cap 1 structure by adding a methyl group at the 2′-O-position of the second nucleotide from the new 5′ end. Cap 1 rather than cap 0 was used because it confers a higher translation efficiency [132]. The 130-nt capped RNA was polyadenylated with poly(A) polymerase, and both mRNA species were purified by phenol extraction and desalted. The structures of the mRNA molecules were confirmed by polyacrylamide gel electrophoresis and MALDI-TOF mass spectrometry.



Scheme 10 Solid-phase synthesis of RNA by the CEM method (*i*), monophosphorylation at the 5′ end with **1** or **2** (*ii*), and pyrophosphorylation with **5** (*iii*) [129]

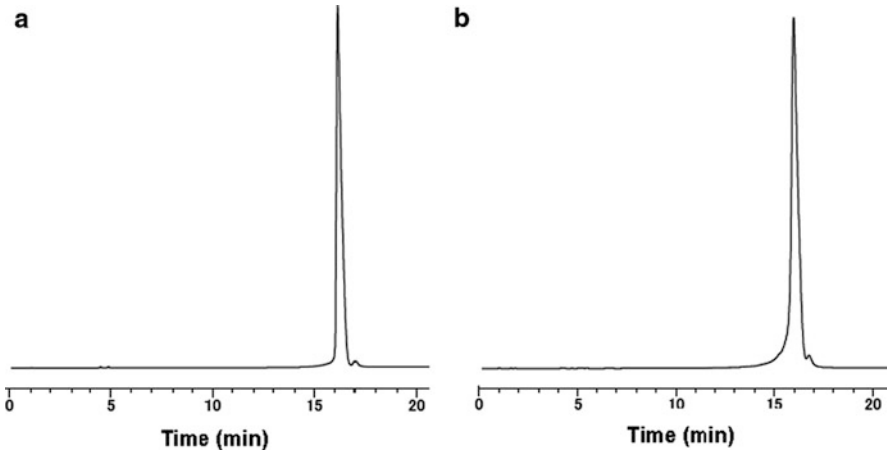


Fig. 18 Anion-exchange HPLC of purified 5′-pyrophosphorylated RNA 130mer (**a**) and 170mer (**b**) [129]

6.4.5 Biological Activity

To assess the biological activity of the artificial mRNA, GLP-1-expression experiments were performed in a cell-free protein synthesis system and in whole cells, and sandwich ELISA was used to determine GLP-1. A eukaryotic cell-free protein synthesis system based on wheat germ lysate was used to express GLP-1 peptide. The negative control was mRNA derived from a 127-nt sequence that was identical to the 130mer except that it lacked the second codon, so that it had a disrupted Kozak sequence. mRNA derived from the 127mer was shown to have the cap 1 structure and a poly(A) tail of about 60 nt. As expected, the negative control hardly supported GLP-1 expression, whereas mRNA derived from both the 130mer and the 170mer supported GLP-1 expression in a dose-dependent manner (Fig. 19). These results demonstrate that artificial mRNA derived from chemically synthesized RNA functioned in vitro. Furthermore, mRNA derived from the 130mer, whose poly(A) tail, at an average length of 80 nt, was about twice as long as that of the 170-nt mRNA, supported GLP-1 expression slightly better than the 170mer did (Fig. 19).

To test the ability of the artificial mRNA to support GLP-1 expression in whole cells, Chinese hamster ovary cells were transfected with artificial mRNA by electroporation. Twelve hours after transfection, cells were harvested and GLP-1 was determined by sandwich ELISA. mRNA derived from both the 130-nt and the 170-nt RNA supported GLP-1 expression, albeit weakly, whereas control RNA with no cap 1 or poly(A) tail did not.

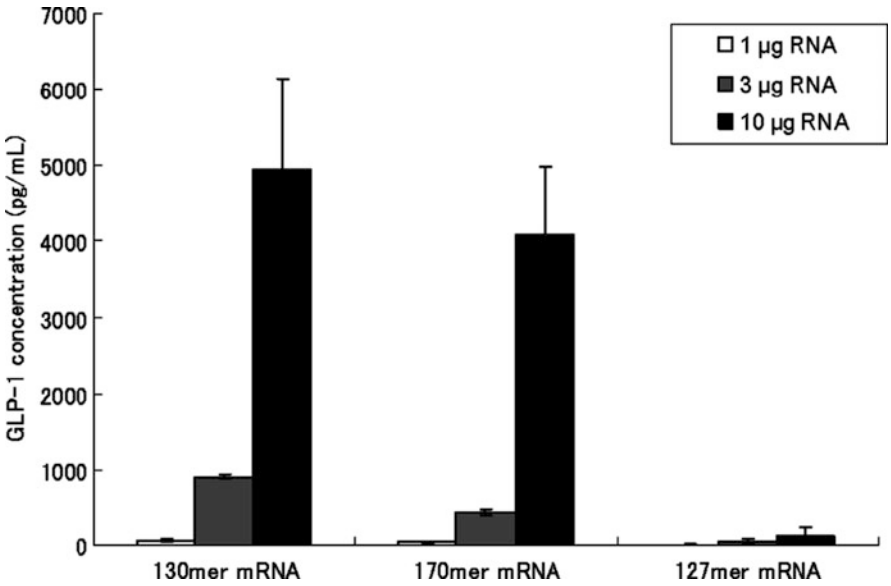


Fig. 19 GLP-1 expression of artificial mRNA in vitro. Artificial mRNA was added to a wheat germ cell-free protein synthesis system, and GLP-1 expression determined by sandwich ELISA. Each value is the average of three independent experiments, and the error bars indicate the standard deviation [129]

6.4.6 Summary

A designed 130-nt mRNA sequence encoding GLP-1 was synthesized by the CEM method. The 130mer was 5′-pyrophosphorylated with a novel reagent, capped, and enzymatically polyadenylated. A 170-nt mRNA was also prepared that had a chemically synthesized 40-nt poly(A) tail instead of the enzymatically added poly(A) tail. These artificial mRNA molecules supported GLP-1 production in a dose-dependent manner in a cell-free protein synthesis system. The technology for synthesizing mRNA described here can provide access to freely designed mRNA for basic and applied research. In addition, the study extends to 170 nt the length of RNA oligomer known to be capable of being synthesized by the CEM method.

7 Concluding Remarks

In this chapter, we have focused on antisense RNA oligomers and described their synthesis and stereospecific backbone modification, two of the basic technologies required for their application to research and drug development. We have developed a practical new RNA synthetic method that has particular advantage in the

synthesis of very long RNA oligomers. The method is based on the use of CEM as a 2′-hydroxyl protecting group that is readily removable by fluoride anion under mild conditions, and it allows RNA to be synthesized more simply and easily than by the conventional methodology. Using the CEM method, we have synthesized RNA oligomers with a yield and purity comparable to that obtained in DNA synthesis, and we have used the method to demonstrate chemical approaches to shRNA, dshRNA, pre-miRNA, and mRNA synthesis. Our progress in the synthesis of RNA oligomers of more than 50 nt in length opens new approaches to RNA therapeutics such as synthetic shRNA and dshRNA, while synthetic pre-miRNA promises to be useful for the functional analysis of noncoding RNA. We have also introduced our chemical approach to mRNA synthesis, demonstrating that the CEM method is capable of handling the synthesis of RNA oligomers up to at least 170 nt in length. We have synthesized RNA oligomers with stereospecific phosphorothioate or boranophosphate linkages and found that the *R*p configuration of phosphorothioates and the *S*p configuration of boranophosphates showed better thermal stability than natural RNA oligomers in the RNA–RNA binding interaction. These and other physicochemical properties of RNA–RNA complexes with stereospecific backbone modifications might influence their biological activity, so that stereospecific backbone modification should be an important consideration in the design of RNA therapeutics. We hope that the application of our work to research on functional RNA will help pave the way for new therapeutic applications of antisense nucleic acids.

Acknowledgments Although most of the research described in this chapter was carried out in our laboratories, we are pleased to introduce in Sect. 5.2 some of the excellent work carried out by Professor Takeshi Wada and his coworkers at the University of Tokyo. We thank Professor Wada for his kind permission to include this material. We are grateful to all of the individual researchers who carried out the work and to those who helped in the preparation of the manuscript, including Dr. Kazuchika Takagaki, Mr. Toshihiro Ueda, Dr. Hidetoshi Kitagawa, Messrs. Hirofumi Masuda, Seigo Nagata, and Kimihiko Higashiyama, and Ms. Yukiko Enya. We are especially grateful to Dr. Makoto Miyagishi of the National Institute of Advanced Industrial Science and Technology (AIST), Tsukuba, Japan, for his careful reading of the manuscript.

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Development and Modification of Decoy Oligodeoxynucleotides for Clinical Application

Mariana Kiomy Osako, Hironori Nakagami, and Ryuichi Morishita

Abstract Several chemical and structural modifications have been employed to improve the therapeutic effectiveness of oligodeoxynucleotides (ODNs), which largely depends on their stability against nucleases, specificity to the target nuclear factor, and efficient cellular and tissue uptake. Phosphorothioation of ODNs has been used in order to decrease their susceptibility to degradation by exo- and endonucleases. To overcome problems related to safety and the cost of production resulting from the fully chemical modified ODN, we developed a non-chemically modified decoy ODN, the ribbon-type decoy, by ligation of the extremities of two single phosphodiester strands. Moreover, we developed a chimera decoy with binding sequence for two different transcription factors. In this review we also discuss briefly the use of a biodegradable polyester as a carrier of ODN.

ODN act as a decoy for specific transcription factors, and is used to attenuate the authentic *cis–trans* interaction, leading to removal of *trans*-factors from the endogenous *cis*-elements and subsequent modulation of gene expression. We developed an ODN that targets nuclear factor kappa B (NF-κB), which plays a pivotal role in the coordinated activation of inflammatory cytokines and expression of adhesion proteins; and chimera decoys for both NF-κB and Ets, and for both NF-κB and E2F.

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We discuss some studies on NF-κB decoy ODN and chimera decoys in cardiovascular and bone disease in mice, rat, and dog animal models, as well as results from some clinical trials.

Keywords Bone disease, Cardiovascular disease, Decoy oligodeoxynucleotide, NF-κB

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1 Decoy Oligodeoxynucleotides

Decoy oligodeoxynucleotides (ODN) are synthetic double-stranded oligodeoxynucleotides that act as decoy *cis*-elements to block the binding of transcription factors to promoter regions of targeted genes, resulting in the inhibition of gene expression. Decoy ODN strategy is particularly attractive for several reasons: the potential drug targets (transcription factors) are plentiful and readily identifiable; knowledge of the exact molecular structure of the target transcription factor is unnecessary; and the synthesis of the sequence-specific decoy is relatively simple and can be targeted to specific tissues.

Therapeutic effectiveness of synthetic double-stranded ODNs in modulating specific gene expression largely depends on several factors, including stability, specificity, and the efficient cellular and tissue uptake of ODN. One of the obstacles in decoy ODN strategy as a pharmaceutical drug concerns the stability of ODN in cells and blood.

Since the phosphodiester ODN, the natural type (N-ODN), is precluded due to its instability under physiological conditions, chemical modifications such as phosphorothioation (S-modification) and methylphosphonation were employed in order to decrease the susceptibility of ODN to degradation by exo- and endonucleases. The first generation of chemically modified decoy is the phosphorothioated ODN (S-ODN), which consists of the replacement of a nonbridging oxygen for sulfur in the phosphate group of the deoxynucleotide backbone (Fig. 1). Moreover, we developed the phosphorothioated chimera decoy with binding sequences for two different transcription factors.

Although the efficacy of the first generation of phosphorothioated ODN in inhibiting a large variety of transcription factors has been reported [1–3], the use of S-ODN has brought other problems, such as safety and the cost of production

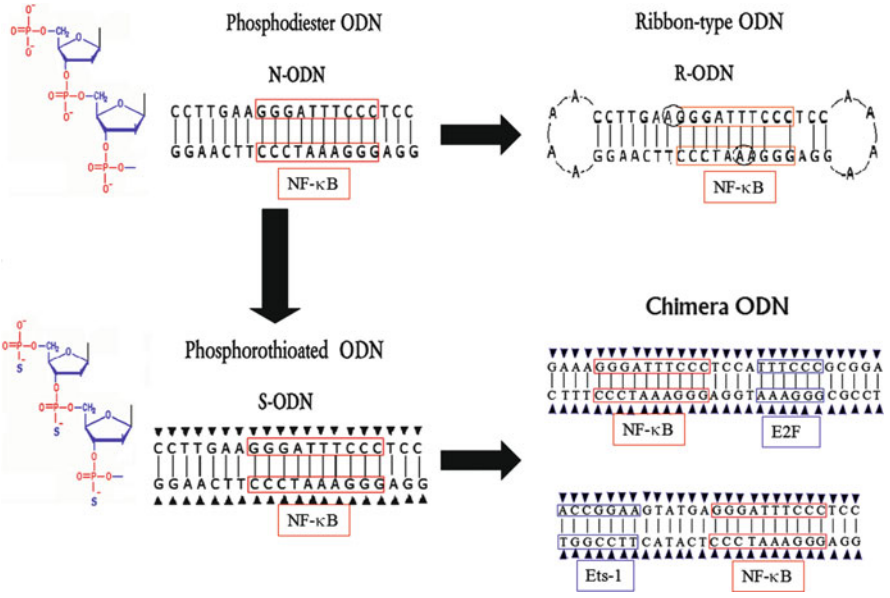

Fig. 1 Non-modified ODN (*N-ODN*), phosphorothioated ODN (*S-ODN*), ribbon-type ODN (*R-ODN*), and chimera decoy. The NF-κB, Ets-1, and E2F binding sequences are marked by *rectangles*. *Filled triangles* in the S-ODN represents the phosphorothioation, and *circles* in the R-ODN indicate the bases involved in the ligation reaction

resulting from that chemical modification [4–7]. One of the major concerns is the generation of nonspecific effects, particularly those attributed to the polyanionic nature of S-ODN. Non-sequence-specific inhibition may operate through blockade of cell surface receptor activity or interference with other proteins [8]. The toxicity of S-ODN may also be relevant. Although low dosage administration does not seem to cause any toxicity, bolus infusions could be dangerous. High doses over prolonged periods of time may cause kidney damage, as evidenced by proteinuria and leukocytes in urine in animals; liver enzymes may also be increased in animals treated with moderate to high doses [9]. Several S-ODN have been shown to cause acute hypotensive events in monkeys [10, 11], probably due to complement activation [12]. More recently, prolongation of prothrombin, partial thromboplastin, and bleeding times has been reported in monkeys [13].

Although those effects are transient if managed properly, and are relatively uncommon, a new construction called ribbon-type ODN (R-ODN) was designed to overcome nonspecific effects caused by chemical modifications. This is a non-chemically modified decoy ODN with a dumbbell-shaped structure formed by ligation of the extremities of two single phosphodiester strands. Such construction significantly increased the stability of the phosphodiester backbone against nucleases compared to N-ODN, and was as efficient as S-ODN in binding to the transcription factor NF-κB [14]. Even though phosphorothioation has been considered to decrease the S-ODN specificity, it increases the stability against nucleases.

On the other hand, the dumbbell-shaped structure does not interfere with the specificity of R-ODN, but is still less stable in serum than S-ODN; incubation with 5% fetal bovine serum for 24 h completely degraded R-ODN, while S-ODN remained almost intact [14].

The development and optimization of ODN construction is a growing field, with new constructions emerging fast and with justified expectations for therapeutic applications. Partially chemically modified R-ODN is expected in the near future.

Transcription factors are key players in disease pathology. They recognize a specific sequence relatively near the site where gene transcription starts, and switch the target gene expression on or off. Therefore, inhibition of a specific transcription factor may prevent the expression of multiple genes responsible for the disease development. In this section we introduce the transcription factors NF-κB, Ets-1, and E2F, and some successful uses of decoy ODN targeting NF-κB in animal models.

2 NF-κB Decoy ODN

2.1 NF-κB

NF-κB (nuclear factor-kappa B) is a transcription factor so named because its first identified binding site is located within an enhancer in the Ig κ light chain gene in mature B cells. The functional NF-κB is a homo- or heterodimer of homologous proteins that share a common structure motif called the Rel domain. The Rel family in the vertebrate includes five cellular proteins: p50 (NF-κB1), p52 (NF-κB2), p65 (RelA), RelB, and c-Rel. The most common NF-κB consists of a p65:p50 heterodimer, but there exists a variety of homodimers and heterdimers, each of which activates its own characteristic set of genes.

NF-κB activation is triggered by signal-induced phosphorylation of specific serine residues in the inhibitor κB (IκB) proteins by an enzymatic complex called IκB kinase (IKK), which is activated by TNFR (tumor necrosis factor receptor), TCR (T cell receptor), or cytokines receptors and involves TRAF (TNFR-activated factor) family adapter proteins. The phosphorylation targets the inhibitor for ubiquitylation and rapid degradation by proteasome and, as a consequence of the release from IκB, NF-κB translocates into the nucleus and binds to specific sequences in the promoter region, called κB-sites, which regulate the expression of target genes. The gene expression controlled by NF-κB regulates cell growth and differentiation, inflammatory responses, apoptosis, and neoplastic transformation.

NF-κB is activated by a variety of stimulants: reactive oxygen intermediates, hypoxia, hyperoxia, cytokines, protein kinase C activators, mitogen-activated protein kinase (MAPK) activators, dsRNA, UV radiation, and bacterial or viral products such as lipopolysaccharide.

Due to the role of NF-κB as a convergent point for the pathways of different stimulants, this transcription factor has a key role in many pathologies. Therefore, strategies to specifically inhibit NF-κB in a certain organ or tissue, and consequent suppression of multiple gene expression have been pursued.

2.2 NF-κB Decoy ODN in Cardiovascular Diseases

2.2.1 Myocardial Infarction

Myocardial reperfusion injury develops mostly as a result of severe damage to myocytes and endothelial cells, induced by the complex interaction of multiple cytokines and adhesion molecules activated by reperfusion. Increased NF-κB binding activity was confirmed in hearts with myocardial infarction. Transfection of NF-κB decoy ODN into rat coronary arteries before left anterior descending coronary artery occlusion markedly reduced the damaged area of myocytes 24 h after reperfusion, whereas no difference was observed between scrambled decoy ODN-treated and non-transfected rats. The therapeutic efficacy of this strategy via intracoronary administration immediately after reperfusion, similar to the clinical situation, was also confirmed. The selectivity of the NF-κB decoy ODN effect was shown by the reduction of the damaged myocardial area, which was not observed in rats treated with antisense ODN directed against the rat gene encoding inducible nitric oxide synthase. The specificity of the NF-κB decoy in the inhibition of cytokine and adhesion molecule expression was also confirmed by in vitro experiments using human and rat coronary artery endothelial cells (ECs). Transfection of NF-κB decoy ODNs markedly inhibited the protein expression of cytokines [interleukin (IL)-6 and IL-8] and adhesion molecules (VCAM, ICAM, and E-selectin) in response to TNF-α stimulation in human aortic ECs. In contrast, the control scrambled decoy ODN failed to inhibit the induction of these protein expressions. Cell numbers after transfection were not changed, indicating that the NF-κB decoy induces a specific inhibitory effect rather than nonspecific cytotoxicity [2].

2.2.2 Vascular Bypass Graft Occlusion

Autologous vein is commonly used for the surgical treatment of coronary artery disease and peripheral artery disease. Although bypass grafts are highly successful in relieving symptoms in patients with severe ischemic arterial disease, the long-term survival of vein grafts is still a crucial problem. Acute vein graft failure is mainly due to thrombosis, and late failure is associated with progressive graft atherosclerosis. Another important process involved is the progression of neointimal hyperplasia. It is mainly caused by endothelial injury and subsequent migration and accumulation of blood-derived cells such as macrophages. These cells express numerous growth factors, cytokines, and proteases regulated by

NF-κB activation, ultimately leading to vascular smooth muscle cell (VSMC) migration and proliferation from media into intima. In the vein graft of a rabbit hypercholesterolemic model, transfection of NF-κB decoy ODN, but not scrambled ODN, significantly inhibited the migration and accumulation of macrophages in the subendothelial layer, and inhibited VSMC growth by induction of VSMC apoptosis. Moreover, there was an inhibition of the transformation of matrix metalloproteinase (MMP)-9 into active MMP-9, and reduced MMP-2 activity. Transfection of NF-κB decoy ODN resulted in the preservation of acetylcholine-mediated vasorelaxation [15]. Taken together, the inhibition of NF-κB activity by decoy ODN in vein grafts protected the surviving endothelial cells from hemodynamic stress and ischemic injury at the time of surgery.

2.2.3 Restenosis After Angioplasty

NF-κB decoy ODN has also been reported as a potential device in the treatment of restenosis after balloon angioplasty. Intimal hyperplasia, as mentioned above, develops largely as a result of VSMC proliferation and migration induced by the complex interaction of multiple growth factors activated by vascular injury. Transfection of NF-κB decoy ODN into balloon-injured rat carotid artery or porcine coronary artery markedly reduced neointimal formation, whereas no difference was observed between scrambled decoy ODN-treated and untransfected blood vessels [16, 17]. In addition to VSMC proliferation, endothelial damage also contributes to the development of restenosis. Interestingly, transfection of NF-κB decoy ODN inhibited EC death, and consequently decreased vascular inflammation because ECs play an important role in suppression of VSMC growth, maintenance of vascular tonus, and protection from monocyte and platelet adhesion.

On the basis of the therapeutic efficacy of this strategy, we obtained permission for a second clinical trial (starting in 2002) using the decoy strategy to treat restenosis. In this trial, NF-κB decoy ODN was delivered to the vessel wall through a hydrogel-coated catheter without any viral or nonviral vector. Efficient ODN transfection was confirmed with fluorescein isothiocyanate (FITC)-labeled ODN. The hydrogel-coated catheter was able to deliver the ODN not only to the coronary endothelium, but also to the vascular wall [18].

We have recently prepared an open-label phase I/IIa clinical trial (the INDOR study; a phase I/IIa open-label multicenter study to assess the inhibitory effects of NF-κB decoy ODN on restenosis after stenting in coronary artery) to evaluate the safety and efficacy of NF-κB decoy ODN. Seventeen patients were treated with NF-κB decoy ODN after percutaneous coronary intervention (PCI) using bare metal stents. As a result, the stenosis improved to $1.4 \pm 5.9\%$ after the intervention. Serum monocyte chemotactic protein-1 (MCP-1) levels were significantly suppressed in NF-κB decoy ODN-treated patients on day 3 after the PCI. Significant restenosis was found in only one of the 17 patients after 6 months, and the average restenosis rate was $39.6 \pm 22.3\%$. No in-stent thrombosis was found and no

significant systemic adverse effect occurred in any of the patients during this observation period [19].

Although further placebo control trials are required, the aforementioned results suggest the clinical usefulness and safety of the NF-κB decoy ODN to prevent restenosis.

2.2.4 Cardiac Transplant Rejection and Vasculopathy

Acute rejection and graft arteriopathy limit the long-term survival of recipients after cardiac transplantation. Acute rejection is enhanced by several cytokines, adhesion molecules, and myosin heavy chain expression, and the arteriopathy is characterized by intimal thickening comprised of proliferative VSMCs. NF-κB decoy ODN was infused into donor hearts in a complex with HVJ (Hemmagluttinating virus of Japan)-AVE-liposome and transplanted into murine recipients. Nontreated ($n = 6$; 7.8 ± 0.4 days) or scrambled decoy-transfected ($n = 6$; 8.0 ± 0.6 days) allografts were acutely rejected, whereas NF-κB decoy transfection significantly prolonged allograft survival ($n = 6$; 13.7 ± 2.4 days, $P < 0.05$). In addition, NF-κB decoy not only attenuated myocardial cell infiltration, but also inhibited arterial neointimal formation in cardiac allografts [20].

2.2.5 Chimera Decoy ODN Against NF-κB and Ets-1 in Abdominal Aorta Aneurism

NF-κB inhibition by decoy ODN has also been reported in a rat model for abdominal aorta aneurism (AAA) using a chimeric decoy ODN with binding sites for two transcription factors: NF-κB and Ets. Destruction of elastin is considered to be one of the major causes of abdominal aortic aneurysm (AAA). Elastic fibers normally maintain the structure of the vascular wall against hemodynamic stress. Proteolytic degradation induces remodeling of the extracellular matrix, resulting in aneurysmal development and, finally, ruptures in the vascular wall. MMP secreted by invasive macrophages, migrating VSMCs, and ECs play important roles in such mechanisms of AAA. NF-κB regulates the transcription of MMP-1, MMP-2, MMP-3, and MMP-9. The Ets family activates the transcription of genes encoding MMP-1, MMP-3, MMP-9, and urokinase plasminogen activator; all of which are proteases involved in extracellular matrix degradation.

AAA was induced in rats by transient aortic perfusion with elastase, and the decoy ODN was transfected by wrapping a delivery sheet containing the chimeric decoy ODN around the aorta. The prevention of aortic dilatation was confirmed by histological studies, and the progression of AAA was inhibited by the chimeric ODN, even 4 weeks after transfection. There was marked inhibition of elastin proteolysis and macrophage migration, and a decrease in MMP gene expression as compared with scrambled decoy ODN and the non-transfected group [15].

2.2.6 Chimera Decoy ODN Against NF-κB and E2F in Anastomotic Intimal Hyperplasia

Prosthetic grafts are commonly used for infrainguinal bypass grafting to treat arterial occlusive disease, but long-term patency remains a crucial problem. One major limitation of prosthetic bypass grafting is the usage of small diameter vessels, such as tibial or peroneal artery bypass, and the graft failure is mainly due to the progression of anastomotic intimal hyperplasia, which causes significant lumen narrowing. The luminal lining of the prosthetic graft is composed of pseudointima and lacks ECs. The lack of an endothelial monolayer disrupts the homeostatic regulation of thrombosis, platelet activation, and leucocyte adhesion, resulting in VSMC proliferation and migration. Therefore, inhibition of VSMC proliferation is thought to prevent progression of intimal hyperplasia, and E2F has attracted attention in this process because it is a pivotal cell-cycle transcription factor.

We developed a chimera decoy strategy to inhibit both NF-κB and E2F simultaneously. Treatment with chimera decoy ODN could reduce the inflammatory response as well as VSMC proliferation and migration in the process of neointimal formation of anastomotic intimal hyperplasia in the prosthetic graft placement in a rabbit hypercholesterolemia model. Chimera decoy ODN also accelerated re-endothelialization. Expression of platelet-derived growth factor (PDGF)-BB and PDGF receptor-β were also suppressed and resulted in a reduction of VSMC accumulation. In addition, chimeric decoy ODN treatment inhibited macrophage accumulation, which was accompanied by a reduction in gene expression of vascular cell adhesion molecule-1 and monocyte chemoattractant protein-1 [21].

2.3 NF-κB Decoy ODN in Bone Disease

2.3.1 Rheumatoid Arthritis

Rheumatoid arthritis (RA) is a chronic inflammatory disease characterized by articular inflammation in which the osteoclasts generated within the synovial membrane are probably involved in bone destruction in vivo. Osteoclasts are activated by both macrophage colony-stimulating factor (M-CSF) and receptor activator of NF-κB ligand (RANKL). A crucial target of RANKL is the activation of NF-κB, and transfection of R-ODN decoy against NF-κB into osteoclasts decreased the RANKL-induced activation, as measured by TRAP staining and pit formation assay. The transfection also decreased the expression of the NF-κB target gene *NFATc1*, which is a master switch for regulating the terminal differentiation of osteoclasts. Using the collagen-induced arthritis animal model, we found that transfer of R-ODN into rat ankle joints resulted in marked improvement in arthritis score compared to S-ODN and control groups, and significantly decreased the osteoclast number and activity in the joint as measured by hematoxylin and eosin staining and TRAP staining [22].

2.3.2 Periodontal Diseases

As previously mentioned, NF-κB is involved in osteoclast differentiation and activation, and the blockade of the NF-κB pathway is a potential therapeutic strategy for treating bone diseases such as periodontitis, which is a subgingival inflammation caused by bacterial infection. NF-κB decoy ODN was topically applied for experimental periodontitis in a debris-accumulation model and for wound healing in a bone-defect model of beagle dogs. Application of S-ODN significantly reduced IL-6 activity in crevicular fluid and improved alveolar bone loss, as shown by analysis of dental radiographs and dual-emission X-ray absorptiometry (DEXA) scans. Direct measurement of exposed root that had lost alveolar bone support revealed that NF-κB decoy treatment dramatically protected bone from loss through the inhibition of osteoclastic bone resorption, and promoted the healing process as compared with scrambled ODN, as shown by micro-computer tomography (CT) analysis [23].

2.3.3 Osteoporosis

Exaggerated osteoclast activation leads to another bone disease – osteoporosis. In an ovariectomized rat model, the estrogen deficiency induces osteoporosis, which was reversed by continuous administration of NF-κB decoy using an osmotic pump. The reversal was indicated by attenuation of TRAP activity, significant increase in calcium concentrations in the femur and tibia, and a decrease in urinary deoxypyridinoline. In agreement, NF-κB decoy ODN infusion in an osteoporosis model of vitamin C-deficient rat dramatically improved the bone length, weight, and mineral density, as assessed by DEXA [24].

3 Perspectives for Decoy ODN Strategy

Decoy ODN-based therapy still shows some unsolved issues. Further modifications of ODN will facilitate the potential clinical utility of these agents by, for example, (1) increasing its half-life, which will allow a shorter intraluminal incubation time to preserve organ perfusion and prolong the duration of biological action; (2) improvement of uptake of ODN by a specific target cell using efficient vehicles or innovations in the gene transfer methods, in a way that the nonspecific effects of high doses can be avoid; and (3) improvement of ODN resistance against nucleases by chemical or structural modifications, because the site of decoy ODN effects is the nucleus and because bypassing the endocytotic pathway and translocation from the cytoplasm are crucial for therapeutic efficiency.

Recently, studies with the biodegradable polyester poly(DL-lactide-*co*-glycolide) (PLGA) showed that PLGA microspheres encapsulating NF-κB decoy ODN were

able to release the ODN at a constant rate for about 40 days [25]. In the chronic inflammation induced by a sponge implant model in rats, injection of decoy ODN–microspheres into the sponge significantly decreased leukocyte infiltration and granuloma formation for up to 15 days after implantation, through inhibition of NF-κB activation. The authors argue that the combination of long-term release and efficient protection of the encapsulated decoy ODN against nucleases could explain the effect obtained [26].

More studies concerning the release rate, ODN loading density, and control of local concentrations at delivery sites are expected, as well as further studies on biodegradable polymers and additional modifications of the ODN itself. In the future, ODN-based gene therapy might overcome present limitations and offer an alternative for treatment of unmet diseases.

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Modulation of Endosomal Toll-Like Receptor-Mediated Immune Responses by Synthetic Oligonucleotides

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Abstract Toll-like receptors (TLRs) 7, 8, and 9 belong to a family of endosomal receptors that are known to detect pathogen-associated nucleic acid molecular patterns and induce appropriate immune responses. Viral single-stranded RNA is the ligand for TLR7 and TLR8, and bacterial and viral DNA containing unmethylated CpG motifs is the ligand for TLR9. We have extensively studied the structure–activity relationships of synthetic oligonucleotides towards the goal of creating novel agonists of TLRs 7, 8, and 9 to modulate immune responses mediated through targeted receptors. Agonists of TLRs 7, 8, and 9 are being studied as therapeutic agents for various diseases, including cancers, allergies, asthma, and infectious diseases, and also as adjuvants with vaccines. In addition, under certain pathological conditions, TLR7 and TLR9 are shown to recognize immune complexes containing self-nucleic acids and contribute to the progression of autoimmune diseases, including lupus, arthritis, psoriasis, and multiple sclerosis. Using the insights gained by studying the interactions of oligonucleotides with endosomal TLRs, we have created oligonucleotide-based antagonists that inhibit both TLR7- and TLR9-mediated immune responses for the treatment of autoimmune and inflammatory diseases. Out of the large portfolio of oligonucleotide-based TLR agonists and antagonists designed, four candidates are currently being evaluated in clinical trials for a broad range of disease indications.

Keywords Agonist · Antagonist · Immune-modulatory oligonucleotides · Immune stimulation · Oligodeoxynucleotides · Oligonucleotides · Oligoribonucleotides · Toll-like receptor 7 · Toll-like receptor 8 · Toll-like receptor 9

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Abbreviations

ADCC	Antibody-dependent cellular cytotoxicity
AE	Adverse event
CMI	Cell-mediated immunity
CpG	Cytidine-phosphate-guanosine
DC	Dendritic cell
ds	Double-stranded
EGFR	Endothelial growth factor receptor
HEK	Human embryonic kidney
HIV	Human immunodeficiency virus
IFN	Interferon
IL	Interleukin
IMO	Immune-modulatory oligonucleotide
IP-10	IFN-γ-inducible protein 10
IRAK	IL-1-associated kinase
LPS	Lipopolysaccharide
LTR	Long terminal repeat
mDCs	Myeloid dendritic cells
MyD8	Myeloid differentiation factor 88
NF-κB	Nuclear factor-kappa B
NK	Natural killer
NSCLC	Nonsmall cell lung carcinoma
ODN	Oligodeoxynucleoide

OME	Otitis-mediated effusion
ORN	Oligoribonucleotide
OVA	Ovalbumin
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood mononuclear cell
pDC	Plasmacytoid dendritic cell
PFS	Progression-free survival
PO	Phosphodiester
PRR	Pattern-recognition receptor
PS	Phosphorothioate
RA	Rheumatoid arthritis
s.c.	Subcutaneous
SIMRA	Stabilized immune modulatory RNA
ss	Single-stranded
TERT	Telomerase reverse transcriptase
Th1	T helper type 1
TIR	Toll/interleukin-1 receptor
TLR	Toll-like receptor
TRAF6	TNF receptor-associated factor 6
TRIF	TIR domain-containing adaptor-inducing interferon-β
VEGF	Vascular endothelial growth factor

1 Introduction

The innate immune system is the first line of defense for recognition of danger signals associated with pathogens, this then leads to the activation of an immediate, rapid, and appropriate immune responses [1]. Besides limiting the early spread of infection, the innate immune system also primes delayed pathogen- or antigen-specific adaptive immunity and memory responses [2]. The innate immune system consists of highly conserved receptors called pattern-recognition receptors (PRRs), which detect danger signals based on cellular constituents present within the pathogens. Toll-like receptors (TLRs) are one class of PRR, of which ten have now been identified in humans [3]. TLRs recognize pathogen-associated molecular patterns (PAMPs) such as lipoproteins/lipopeptides, flagellin, lipopolysaccharides, and nucleic acids of bacteria and viruses and induce innate immune responses and prime pathogen-specific adaptive immune responses.

2 Toll-Like Receptors

TLRs are transmembrane receptors comprising an extracellular leucine-rich repeat and a cytoplasmic Toll/interleukin-1 receptor (TIR) domain, which are connected through a transmembrane domain [4]. The TIR domain is structurally homologous

to the IL-1 receptor. In general, upon binding to a PAMP, TLRs recruit an appropriate adapter protein to the TIR domain [5]. TLR2–TLR5, and TLR7– TLR9 utilize myeloid differentiation factor 88 (MyD88), and TLR3 and TLR4 utilize TIR domain-containing adaptor-inducing interferon-β (TRIF) as adapter molecules (Table 1). This phenomenon leads to engagement of IL-1-associated kinase (IRAK), TLR-specific additional adapter proteins, and TNF receptor-associated factor 6 (TRAF6) in the signaling pathway, resulting in the activation of transcription factors AP1, ELK1, NF-κB, and the IRFs, depending on the PAMP encountered and TLR activated [5].

Of the TLRs identified in humans, five TLRs, referred to as cell-surface TLRs, are expressed on the cell or plasma membrane and recognize cell-surface constituents such as lipoproteins/lipopeptides (TLR1/TLR2 and TLR2/TLR6), flagellin (TLR5), and lipopolysaccharides (TLR4) of extracellular microbial agents [6]

Table 1 Human TLR expression, common ligands and adaptors used in signaling by each TLR

TLR	Expression	Ligand	Adaptors
Cell surface TLRs			
TLR1	Macrophages, mDCs, pDCs, T cells, B cells, granulocytes	TLR2 coreceptor; PAM3CSK4	–
TLR2	Macrophages, mDCs, pDCs, granulocytes	PAM2CSK4; lipoteichoic acids; MALP-2; Zymosan	MyD88; MAL
TLR4	Macrophages, mDCs, pDCs, T cells, B cells, granulocytes, $CD4^+CD25^+$ Tregs	LPS; Taxol; F protein; fibronectin; HSP60; HSP70; hyaluronan	MyD88; MAL; TRIF; TRAM
TLR5	Macrophages, mDCs, pDCs, $CD4^+CD25^+$ Tregs	Flagellin	MyD88
TLR6	Macrophages, mDCs, pDCs, B cells, $CD4^+CD25^+$ Tregs	TLR2 coreceptor; MALP-2; Zymosan	–
TLR10	Macrophages, mDCs, pDCs, B cells, $CD4^+CD25^+$ Tregs	Not known; TLR2 co-receptor	MyD88
Endosomal TLRs			
TLR3	Macrophages, mDCs, NK cells	Viral dsRNA; polyI.polyC	TRIF
TLR7	pDCs, B cells, granulocytes	Viral ssRNA; imidazoquinolines; nucleoside analogs	MyD88
TLR8	Macrophages/monocytes, mDCs, $CD4^+CD25^{High}$ Tregs	Viral ssRNA; imidazoquinolines	MyD88
TLR9	pDCs, B cells	Viral and bacterial DNA-containing unmethylated CpG motifs	MyD88

TLR Toll-like receptor, *mDCs* myeloid dendritic cells, *pDCs* plasmacytoid dendritic cells, *PAM2-* and *PAM3CSK4* synthetic diacylated lipopeptides, *MALP-2* macrophage-activating lipopeptide 2, *LPS* lipopolysaccharide, *HSP* heat shock protein, *ds* double-stranded, *ss* single-stranded, *MyD88* myeloid differentiation factor 88, *MAL* MyD88 adapter-like, *TRAM* Toll-like receptor adaptor molecule 2, *TRIF* TIR (Toll/interleukin-1 receptor) domain-containing adaptor protein inducing interferon beta; also known as TICAM-1

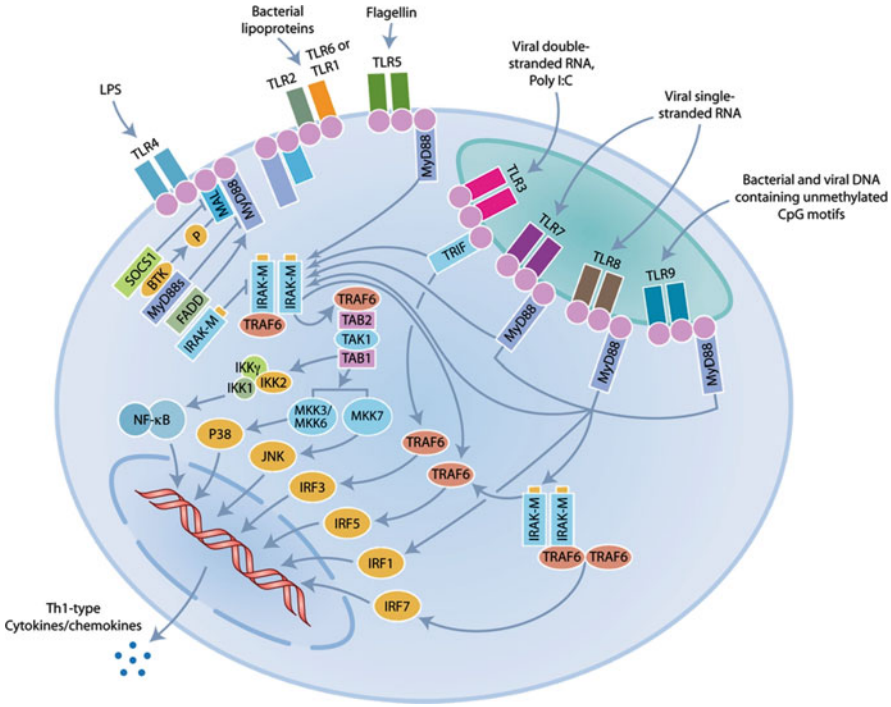

Fig. 1 Localization of TLRs in immune cells, their ligands and a representation of TLR signaling pathways. Nucleic acid-recognizing TLRs 3, 7, 8, and 9 are located in the endolysosomes. Signaling by these receptors upon activation by their respective ligands leads to the activation and nuclear translocation of transcription factors such as the IRFs, NF-κB, and AP1, which promote expression of cytokines

(Table 1). The other four TLRs, referred to as endosomal TLRs, are expressed in the membranes of the endolysosomes and recognize molecular patterns of nucleic acids derived from viral and bacterial pathogens and also synthetic DNA and RNA oligonucleotides, depending on nucleotide composition and structure [3] (Table 1) (Fig. 1). TLR3 is the receptor for viral and synthetic double-stranded (ds) RNAs [7]. TLR7 and TLR8 are the receptors for viral single-stranded (ss) RNAs [8, 9] and imidazoquinolines [10, 11]. Bacterial and viral DNA that contain unmethylated cytidine-phosphate-guanosine (CpG) motifs act as ligands for TLR9 [12] (Fig. 1).

2.1 Endosomal TLRs

TLRs 3, 7, 8, and 9 are the receptors for pathogen-derived nucleic acids. All four are expressed in endosomal membranes, and their expression is cell-specific [13] (Table 1). In humans, TLR3 is expressed in myeloid dendritic cells (mDCs) and

natural killer (NK) cells. TLR7 and TLR9 are expressed in human B cells and plasmacytoid DCs (pDCs). TLR8 is expressed in mDCs, monocytes, and neutrophils. In general, TLR-mediated immune responses include cytokine/chemokine production and upregulation of stimulatory surface molecule expression. However, the cytokine profile produced by activation of each TLR is dependent on the PAMP it recognizes (type of microorganism encountered), the cell type it is expressed in, and the signal transduction cascades the specific TLR activates. In general, nucleic acid-based agonists of endosomal TLRs induce T helper1 (Th1)-type immune responses.

3 Toll-Like Receptors 7 and 8

Single-stranded RNA from viruses such as Influenza, respiratory syncytial, vesicular stomatitis, Sendai, Coxsacki B, Dengue, mouse hepatitis, parechovirus 1, and human immunodeficiency virus induce TLR7- and/or TLR8-mediated immune responses [14, 15]. Short synthetic ssRNAs that mimic viral RNA segments, which are formulated with lipids, act as agonists of TLR7 and TLR8 [8, 9]. Certain small molecules such as imidazoquinoline-based compounds can also act as agonists of TLR7 and TLR8 [10]; however, the interaction between these receptors and imidazoquinolines has not yet been elucidated. Recent studies have shown that imidazoquinolines induce immune responses via activation of other receptors, including A3 adenosine and the opioid growth factor receptors [16, 17]. An imidazoquinoline compound, imiquimod, has been approved for the treatment of actinic keratosis, superficial basal cell carcinoma, and external genital warts by topical administration. Systemic administration of imiquimod is known to cause side effects [18–20]. In addition, certain nucleoside analogs, such as loxoribine, 7-thia-8-oxo-guanosine, and 7-deazaguanosine, have also been shown to induce cytokine production mediated through TLR7 at high micromolar concentrations [11]. Activation of TLR7 requires endosomal localization of the ligands, and the endosomal uptake of nucleoside analogs is not well understood.

3.1 RNA-Based Agonists of TLR7 and TLR8

Recent studies have shown that synthetic ssRNAs and dsRNAs induce immune responses via activation of TLR7 and TLR8 [21–23], and TLR3 [24, 25] respectively. However, systematic studies elucidating which characteristics of RNA are recognized by TLR7 and/or TLR8 are lacking. RNA is rapidly degraded by nucleases in vivo, which limits its use as an immune modulator. Studies reported to date have formulated ssRNA-based TLR7/8 agonists with lipids to increase their nuclease stability and delivery [23, 26].

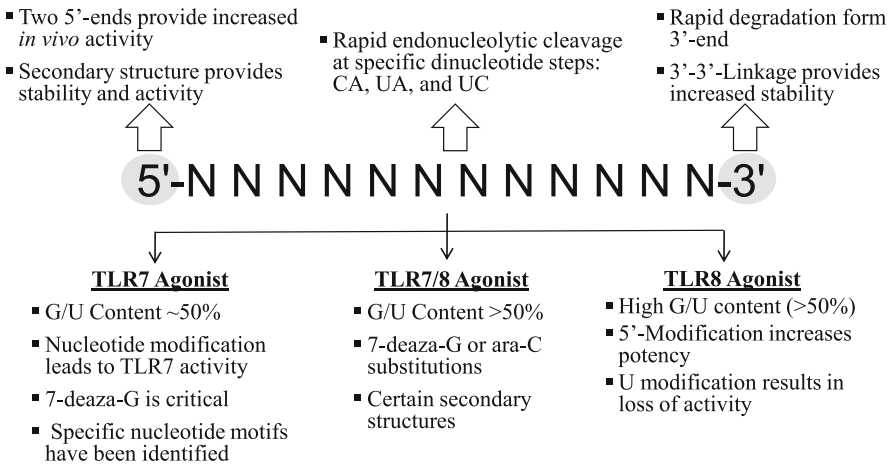

Fig. 2 Summary of structure–activity relationship studies of RNA as agonist of TLR7, TLR8, and both TLR7 and TLR8

To develop metabolically stable RNA for therapeutic applications, we have studied RNAs, referred to as stabilized immune modulatory RNA (SIMRA) compounds, that are linked through their 3′-ends to prevent 3′-exonuclease degradation [27] (Fig. 2). SIMRA compounds are more stable in human serum than are ssRNAs [27, 28]. We have also observed that the stability of ssRNA depends on its nucleotide composition: dinucleotide motifs such as UA, UC and CA are more susceptible to degradation by endonucleases than are other dinucleotides [27, 29]. Therefore, avoiding incorporation of these dinucleotide motifs enhances the stability of ssRNA against endonucleases (Fig. 2).

3.2 TLR7 Agonists

We have designed and synthesized a number of novel SIMRA compounds with varying sequence compositions, substituting 7-deazaguanosine (7-deazaG) for natural guanosine [29] (Fig. 2). Structure–activity relationships of SIMRA compounds have shown that the presence of a 5′-AA/CN (A > C and N = U/C/7-deazaG) and/or C/AUU-3′ (C > A) trinucleotide at the 5′- and 3′-ends of the SIMRA compound and the presence of either a 5′-AN^{1}N^{2}UG1A-3′ [N^1 = A/C; N^2 = U/C/7-deazaG (G1)] or a UG1AZ^{1}G1Z^{2}UU (Z^1 = A<C; Z^2 = C<A) motif confers TLR7 selectivity over other sequence compositions [29]. In immune stimulatory activity studies, TLR7-selective SIMRA compounds induce high levels of interferon-α (IFN-α) production in human pDCs (which express TLR7) with insignificant effect on mDCs (which express TLR8), suggesting TLR7-dependent effects (Fig. 3). The SIMRA compounds that activate TLR7 also induce cytokine production in mice, both

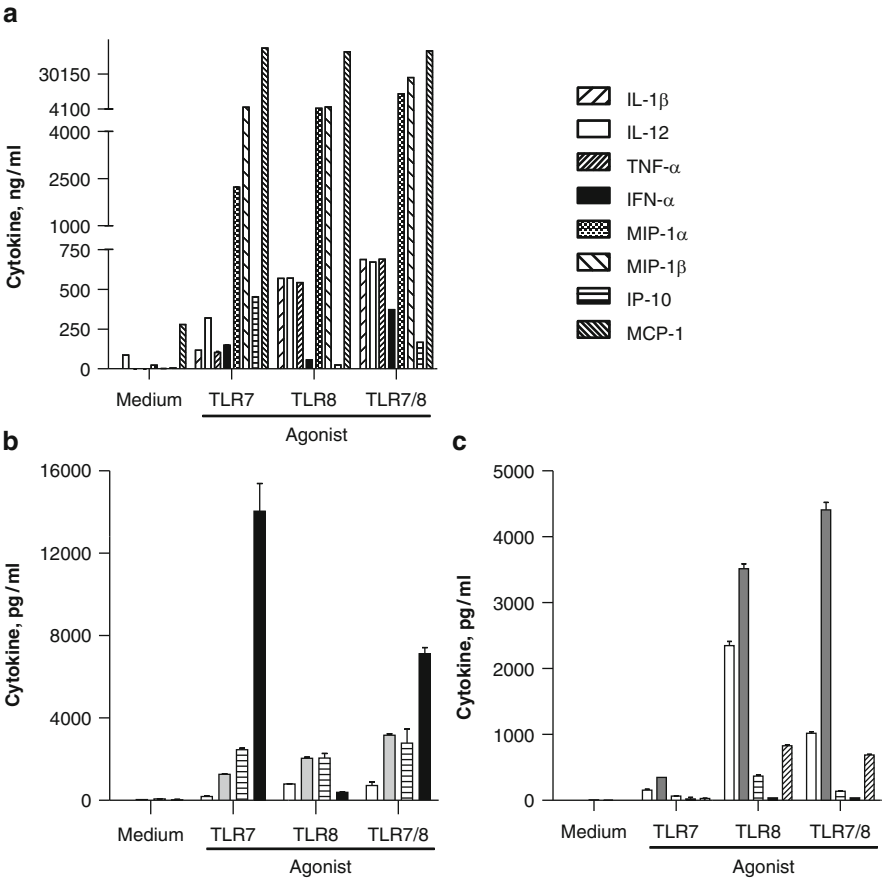

Fig. 3 Cytokine and chemokine induction by representative SIMRA compounds that mediate immune responses via TLR7, TLR8, and both TLR7 and TLR8 in human (**a**) PBMCs, (**b**) plasmacytoid dendritic cells (pDCs), and (**c**) myeloid dendritic cells (mDCs). Freshly isolated human PBMCs, pDCs, and mDCs were cultured with 50 μg/mL (PBMCs) or 100 μg/mL (pDCs and mDCs) SIMRA compounds for 24 h, and the supernatants were assessed for cytokine and chemokine levels by luminex multiplex assay [29–31]. RNA sequences used in the study were: 5′-AAUU**G1**AC**G1**CUU-X-UUC**G1**CA**G1**UUAA-5′ (TLR7 agonist), 5′-YUGCUGCUUGUG-X-GUGUUCGUCGUY-5′ (TLR8 agonist), and 5′-U**G1**CU**G1**CUUCU**G1**-X-**G1**UCUUC**G1**UC**G1**U-5′ (TLR7/8 agonist), wherein X stands for 1,2,3-propanetriol linker, Y for 1,3-propanediol linker, and **G1** for 7-deazaguanosine. All RNAs contained phosphorothioate backbone. Data were adopted from Lan et al. [29–31]

in vitro in splenocytes and in vivo [27, 29]. Importantly, studies in TLR knockout mice show that both TLR7 and MyD88 are required for the immune stimulatory activity of TLR7-selective SIMRA compounds [29]. For the first time we have identified SIMRA compounds that selectively activate TLR7, depending on the sequence composition and chemical modifications incorporated.

3.3 TLR8 Agonists

We have also identified SIMRA compounds that stimulate TLR8 selectively. TLR8-stimulating SIMRA compounds do not require substitution of any chemical modifications [27] (Fig. 2). TLR8 agonists induce high levels of IL-12 and tumor necrosis factor-α (TNF-α) and insignificant amounts of IFN-α [27] (Fig. 3). Incorporation of nonnucleosidic linkers at the 5′-end of SIMRA compounds increases their stability against nucleases and further enhances their ability to activate TLR8 [27].

3.4 Dual TLR7 and TLR8 Agonists

When guanosines in certain TLR8-activating SIMRA compounds are replaced with 7-deazaG, these compounds activate both TLR7 and TLR8, providing unique RNA-based compounds that act as dual TLR7 and TLR8 agonists [27] (Fig. 2). TLR7- and TLR8-activating SIMRA compounds induce dose-dependent Th1-type cytokine production in human PBMCs [27] and IFN-α production in human PBMC and pDC cultures [27] (Fig. 3). pDCs, which express TLR7 and TLR9, but not TLR8, are the primary producers of IFN-α upon stimulation with appropriate TLR agonists [32].

3.5 Effect of Arabinonucleosides in TLR7 and TLR8 Agonists

We have studied substitution of a number of different ribonucleotide modifications in SIMRA compounds for their ability to activate TLR7 and TLR8. Surprisingly, we have found that SIMRA compounds containing arabinoG, arabinoC, arabinoU, or arabinoA substitutions activate TLR8 in transfected HEK293 cell reporter assays. Interestingly, SIMRA compounds containing arabinoC also activate TLR7 and stimulate immune responses in vivo in mice [30]. In human PBMC and pDC cells, SIMRA compounds containing arabinonucleotides induce Th1-type cytokine profiles [30].

3.6 Effect of Secondary Structures in TLR7 and TLR8 Agonists

To study the effect of secondary structures in RNA on TLR7- and TLR8-mediated immune responses, we have designed and synthesized phosphorothioate oligoribonucleotides (ORNs) with self-complementary sequences that form duplex structures with either 3′- or 5′-overhanging sequences [31]. ORNs-containing self-complementary sequences form secondary structures, and the thermal stability

of the duplex depends on the length and GC composition of the duplex [31]. Nuclease stability of ORNs increases with increasing thermal stability of the duplex formed. All ORNs containing a secondary structure show TLR8 activity in transfected HEK293 cells, and induce cytokine and chemokine production in human PBMC cultures [31]. In addition to TLR8 activity, ORNs containing a CUGAAUU motif in the duplex-forming region activate TLR7 in transfected HEK293 cells and induce immune stimulation through TLR7 in human PBMC and pDC cultures, and acutely in mice [31]. These results suggest that secondary structures in ORNs provide nuclease stability and lead to stimulation of immune responses through TLR8 as well as TLR7, depending on the presence of specific nucleotide motifs [31].

3.7 Immune Responses Induced by TLR7 and TLR8 Agonists in Nonhuman Primates

We have studied the immune response profiles induced by acute administration of SIMRA compounds that act as agonists of TLR7 and TLR8 in nonhuman primates in the absence of lipid formulation. Administration of a single-dose of SIMRA compounds to cynomolgus monkeys induces lymphocyte migration; CD69 expression on T lymphocytes, monocytes, and NK cells; and elevation of cytokine and chemokine levels in the plasma [27]. A SIMRA compound that activates both TLR7 and TLR8 induces higher levels of IFN-α than does a TLR8-only-activating SIMRA compound [27]. In these studies, lipid carrier formulations are not needed for the immune response induction observed following administration of SIMRA compounds to nonhuman primates.

4 DNA Containing Specific Nucleotide Motifs Is a Ligand for TLR9

DNA isolates from bacteria elicit antitumor activity [33, 34], augment NK cell activity [35, 36] and induce interferon-α, interferon-β, and interferon-γ production [36]. DNA from different bacterial species, but not mammalian DNA, stimulates proliferation of murine lymphocytes [37]. Further studies showed that synthetic oligonucleotides containing certain self-complementary palindromic sequences with CG dinucleotides, similar to those present in bacterial DNA, increase the cytolytic function of NK cells and induce secretion of IFN-γ [38, 39]. Synthetic oligonucleotides containing CpG motifs were also shown to induce immune responses [40].

For synthetic oligonucleotides to be used as antisense agents, several synthetic modifications have been made to overcome rapid metabolic degradation of the natural DNA backbone. Synthetic oligonucleotides in which one of the nonbridging

oxygens on the phosphate group was replaced with sulfur, called phosphorothioate oligonucleotides, yielded the most promising results as antisense agents. Several of these first-generation phosphorothioate oligonucleotides have been evaluated in human clinical trials for a number of disease indications [41]. Further studies of phosphorothioate oligonucleotides suggest that certain antisense oligonucleotides with phosphorothioate backbones elicit sequence-specific immune responses [42–46]. The sequence-specific immune responses observed with phosphorothioate antisense oligonucleotides and their control sequences are related to the presence of unmethylated CpG-motifs in certain sequence contexts, similar to those reported with bacterial DNA and self-complementary palindromic sequences containing CpG dinucleotides [35, 37, 40]. TLR9 is the receptor that recognizes bacterial and synthetic DNA containing unmethylated CpG motifs and induces immune responses [12].

Stimulation of TLR9 with bacterial and viral DNA containing unmethylated CpG motifs transactivates HIV long terminal repeat (HIV-LTR) and HIV replication [47]. Consistent with these results, we have observed that an antisense oligonucleotide containing a CpG motif and targeted against the gag region of HIV-1, referred to as GEM91, produces dose-dependent increases in viral loads in the plasma of HIV-positive patients compared with placebo control [48]. Though unintended, these results provide proof of concept for biological activity of TLR9 agonists in human studies.

4.1 TLR9 Agonists

Bacterial and synthetic DNAs that contain unmethylated CpG motifs act as agonists of TLR9 and induce Th1-type immune response profiles. The immune-stimulatory effects of TLR9 agonists depend on the nucleotide sequence, nature of the backbone, and the presence of specific structural motifs. Based on the cytokine profiles induced, three distinct classes of TLR9 agonists, referred to as class A/D, B/K, and C, have been reported in the literature [49, 50]. Each class of TLR9 agonist is composed of a different nucleotide sequence that allows formation of structures (or no structures) that generate different immune response profiles [51–59].

4.2 Structure–Activity Relationship Studies of TLR9 Agonists

We have systematically studied the structure–activity relationships of oligonucleotides that act as agonists of TLR9 [60] (Fig. 4). The presence of a CpG motif in oligonucleotides is required for TLR9-mediated immune stimulation. Oligonucleotides with phosphodiester (PO) and phosphorothioate (PS) backbones stimulate TLR9-mediated immune responses [61, 62]. PO oligonucleotides, however, undergo rapid degradation in vivo; therefore PS oligonucleotides, which are

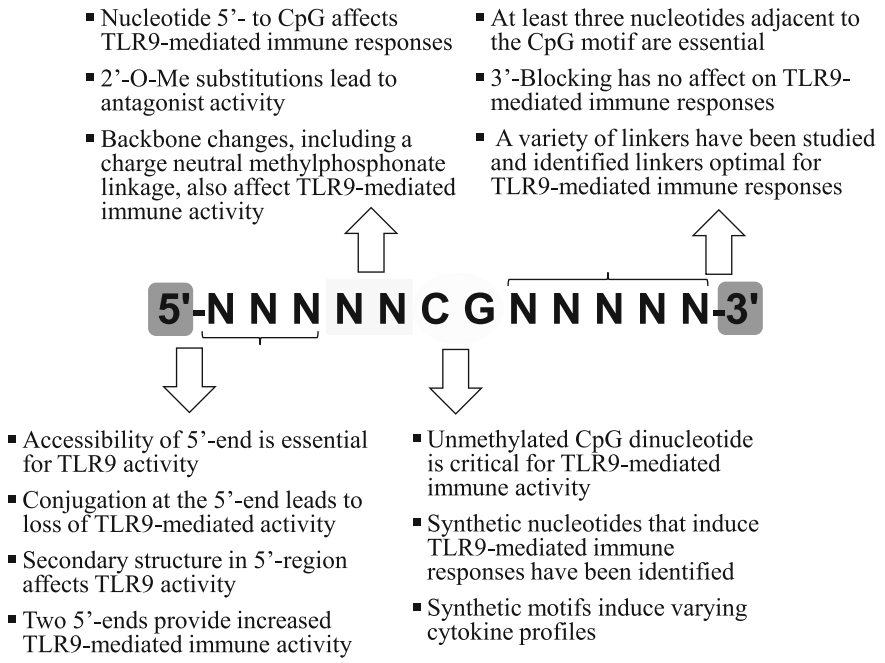

Fig. 4 Summary of structure–activity relationship studies of DNA as agonist of TLR9

less susceptible to degradation by ubiquitous nucleases, are commonly used [63]. Substitution of a sulfur atom for oxygen on the internucleotide PO bond results in the formation of *R*p and *S*p diastereoisomers of the PS bond; the *R*p diastereomer of the PS linkage stimulates a stronger TLR9-mediated immune response than does the *S*p diastereomer [64].

The negative charges on phosphates between and adjacent to cytosine (C) and guanine (G) are also required for TLR9-mediated immune-stimulatory activity (Fig. 4). Neutralization of phosphate backbone charges by incorporation of methylphosphonate linkages at these positions results in the loss of TLR9-mediated immune-stimulatory activity [64–66]. Moreover, TLR9 activation is also dependent on the sequences flanking the CpG dinucleotide, the nature of the nucleotide backbone, and the secondary structures [28, 60, 65, 67, 68].

4.2.1 Role of Sequences Flanking CpG Dinucleotide

Chemical modifications introduced at the 2′-position of the sugar ring of a cytidine or guanosine nucleoside in the CpG motif result in the loss of immune-stimulatory activity of TLR9 agonists [65]. In addition, studies of TLR9 agonists containing chemical modifications such as methylphosphonate linkages [65, 69], 2′-alkyl or 3′-deoxy or -alkyl ribonucleosides [70–72], nonnucleotide linkers [73], or abasic

nucleotides [74, 75] in the flanking sequences indicate that substitutions incorporated at the fourth to sixth nucleotide positions 5′- to the CpG dinucleotide significantly enhance immune-stimulatory activity. By contrast, substitutions incorporated adjacent to CpG on the 5′-side do not show immune responses [65, 70, 71]. In general, modifications incorporated in the 3′-flanking sequence distal to the CpG dinucleotide have effects dependent on the nature of modification [28].

4.2.2 Accessibility of 5′-End of Oligonucleotides Is Essential

We have found that two CpG oligonucleotides linked through their 5′-ends do not induce immune responses despite the presence of two CpG motifs [76, 77]. When the same oligonucleotides are linked through their 3′-ends, they produce higher levels and different types of cytokine profiles than the parent CpG oligonucleotide with a single 5′-end [60, 76–80]. These studies provided the first evidence that an accessible or free 5′-end is required for TLR9-mediated immune stimulation. The presence of two accessible 5′-ends in a TLR9 agonist results in more rapid activation of the transcription factor NF-κB in J774 cells, but a similar activation of MAP kinase, compared with a TLR9 agonist containing a single 5′-end [80].

Studies with different lengths of nucleotides in each segment of TLR9 agonist showed that 11-mers are more active than agonists containing shorter or longer segments [60, 80]. The attachment of oligonucleotides through their 3′-ends not only provides two 5′-ends for optimal activation of TLR9, but also increases their stability against 3′-exonucleases. Oligonucleotides with a PO backbone and as few as five and six nucleotides linked through their 3′-ends act as potent TLR9 agonists to induce immune responses [62, 81]. Moreover, oral administration of a novel TLR9 agonist induces potent mucosal immune responses, acts as an adjuvant with antigens, and prevents and reverses peanut allergy in mouse models due to its greater stability in the gastrointestinal tract [82, 83].

We have shown that blocking the 5′-end of a TLR9 agonist with mono-, di- or oligonucleotides or small-molecule entities, such as fluorescein, abrogates TLR9-mediated immune-stimulatory activity, whereas conjugation of the same at the 3′-end retained the activity [76]. Recently, we have conjugated a model peptide, a 28-amino acid β-amyloid peptide, to either the 5′- or the 3′-end of a TLR9 agonist via C3 and C6 alkyl linkers [84]. Studies of immune-stimulatory activity suggest that TLR9 agonists with peptide conjugated at the 3′-end via C3 or C6 linkers induce immune-stimulatory activity similar to that of the parent TLR9 agonist both in vitro and in vivo in mice [84]. By contrast, conjugation of peptide at the 5′-end of TLR9 agonists significantly abrogates the immune-stimulatory activity, suggesting that conjugation of a peptide or protein at the 3′-end of a TLR9 agonist is optimal and that conjugation at the 5′-end leads to significant loss of TLR9-mediated immune stimulation [84]. Consistent with our findings, a 5′-conjugate of ragweed allergen Amb a 1 to a TLR9 agonist failed to meet the end points in clinical studies [85].

Results of these studies suggest that agonists containing two accessible 5′-ends may facilitate dimerization of the receptor, leading to rapid activation of immune responses [60]. Moreover, TLR9 activation can be modulated through appropriate presentation of the free 5′-ends and synthetic immune-stimulatory motifs, leading to changes in the downstream cytokine induction profiles. In fact, recent studies have shown that TLR9 exists in dimer form and binds to ss-oligonucleotides [86]. However, only oligonucleotides containing a CpG motif cause conformational changes in the endodomain of the receptor, leading to the recruitment of adaptor molecule and subsequent activation of immune-stimulatory signaling pathways [86].

4.2.3 Requirement of Functional Groups on Cytosine and Guanine

As described above, certain chemical modifications introduced within the CpG dinucleotide that alter structure and conformation lead to the loss of immune-stimulatory activity of agonists. One such modification is substitution of the methyl group at the 5-position of cytosine in the CpG motif of TLR9 agonists [66]. Vertebrates use this feature to distinguish self-DNA from that of bacterial DNA, which contains more unmethylated CpG motifs.

We have studied the effects of various pyrimidine analogs (Y), such as 5-OH-dC, dU, dP, 1-(2′-deoxy-β-D-ribofuranosyl)-2-oxo-7-deaza-8-methyl-purine, N^3-Me-dC, N^4-Et-dC, 5-propyne-dC, furano-dT, dF, 4-thio-dU, ψ-iso-dC, and arabinoC in place of cytosine [87–90] (Fig. 5). To understand the role of different functional groups of guanine in the recognition of TLR9, several purine nucleobases (R) such 7-deaza-dG, 7-deaza-8-aza-dG, 9-deaza-dG, N^1-Me-dG, N^2-Me-dG, 2-amino-D-purine, nebularine, 2-amino-dA, 7-deaza-D-xanthine, K-base 6-thio-dG, dI, 8-OMe-dG, 8-*O*-allyl-dG, and arabinoG were examined in place of guanine in the CpG motif [60, 68, 78, 79, 87–90] (Fig. 5). Agonists of TLR9 containing certain cytosine or guanine modifications show activity in TLR9-transfected HEK293 cells, mouse spleen, and human cell-based assays and in vivo in mice. The results provide insight into which specific chemical modifications at cytosine or guanine of the CpG motif are recognized by TLR9 and into the ability to modulate immune responses by substituting natural cytosine or guanine in TLR9 agonists. Moreover, our studies have shown that TLR9 recognizes arabinoC and arabinoG substitutions in cytosine and guanine of a CpG dinucleotide, respectively. More importantly, TLR9 agonists containing specific substitutions induce a different immune response profile to that of a natural CpG dinucleotide [60, 68, 78, 79, 87–90].

4.2.4 Effect of Secondary Structure of TLR9 Agonist

Secondary structures of oligodeoxynucleotides affect TLR9-mediated immune responses. We have carried out studies with TLR9 agonists that form an intramolecular hairpin structure at the 5′-end or the 3′-end for their effect on TLR9-mediated immune responses [91, 92]. These studies suggest that TLR9 agonists

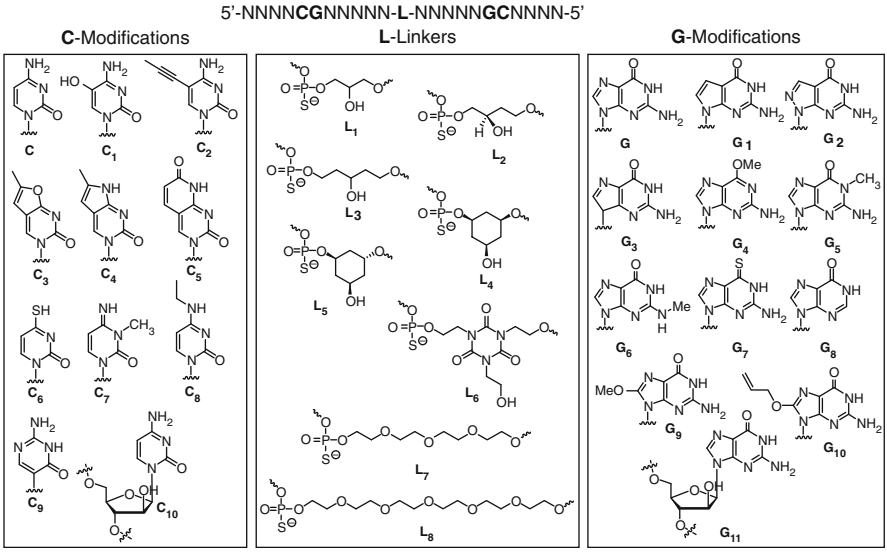

Fig. 5 Structures of various modifications studied for optimal TLR9-mediated immune response. Cytosine modifications: *C* 2′-dC, *C1* 5-hydroxy-2′-dC, *C2* 5-propyne-2′-dC, *C3* furano-2′-dT, *C4* pyrrolo-2′-dC, *C5* dF, *C6* 4-thio-2′-dU, *C7* N^3-methyl-2′-dC, *C8* N^4-ethyl-2′-dC, *C9* Ψ-iso-2′-dC, and *C10* arabinoC. Guanine modifications: *G* 2′-dG, *G1* 7-deaza-2′-dG, *G2* 7-deaza-8-aza-2′-dG, *G3* 9-deaza-2′-dG, *G4* 6-*O*-methyl-2′-dG, *G5* N^1-methyl-2′-dG, *G6* N^2-methyl-2′-dG, *G7* 6-thio-2′-dG, *G8* 2′-deoxyinosine, *G9* 8-*O*-methyl-2′-dG, *G10* 8-*O*-allyl-2′-dG, and *G11* arabinoG. Linkers (*L*): *L1* glycerol (1,2,3-propanetriol), *L2* (*S*)-(−)-1,2,4-butanetriol, *L3* 1,3,5-pentanetriol, *L4* *cis, cis*-1,3,5-cyclohexanetriol, *L5* *cis,trans*-1,3,5-cyclohexanetriol, *L6* 1,3,5-tris(2-hydroxyethyl) isocyanurate, *L7* tetraethyleneglycol, and *L8* hexaethyleneglycol

containing a hairpin structure at the 3′-end show TLR9-mediated immune responses in cell-based assays [91, 92]. Moreover, TLR9 agonists containing a 3′-hairpin structure induce higher levels of IFN-α in human PBMC and pDC cultures, suggesting that TLR9 agonists containing a secondary structure induce higher levels of IFN-α [91, 92]. By contrast, TLR9 agonists containing a hairpin structure at the 5′-end show loss of immune-stimulatory activity [91, 92]. These results are consistent with the concept that an accessible 5′-end is required for optimal activation of TLR9 and that blocking the 5′-end with a secondary structure leads to loss of TLR9-mediated immune responses. However, in vivo studies of 3′-hairpin-containing TLR9 agonists in mice and nonhuman primates suggest that no immune responses are induced (Kandimalla and Agrawal, unpublished results). This observation suggests that TLR9 agonists that can form intramolecular secondary structures, such as hairpins, are not effective inducers of immune responses in vivo.

Oligodeoxynucleotides containing a CpG motif and a palindromic sequence that allows formation of a duplex structure, referred to as class C CpG oligos, have been shown to act as agonists of TLR9 and induce higher levels of IFN-α compared to sequences without a secondary structure [57–59]. However, palindromic sequence containing oligodeoxynucleotides can form intramolecular hairpin structures,

depending on the length and base composition of the palindrome, rather than intermolecular duplex structures. Formation of intramolecular hairpin structure leads to loss of immune stimulatory activity in vivo, as discussed above.

We have designed TLR9 agonists with self-complementary sequences that would preferentially form an intermolecular duplex structure, but not an intramolecular hairpin structure. Our studies have shown the ability of such self-complementary sequences containing TLR9 agonists to induce higher levels of IFN-α production along with other Th1-type cytokines in vitro in human cell-based assays, and higher and sustained levels of IFN-α and IP-10 (IFN-γ-inducible protein 10) induction and NK cell activation in vivo in nonhuman primates [28, 79].

We have evaluated three highly effective IFN-α-inducing agonists of TLR9 to determine the type of duplex structures formed and their ability to induce immune responses in vitro and in vivo in mice and nonhuman primates [93]. Thermal melting studies have shown that, depending on the oligonucleotide sequence composition, they form intramolecular duplexes, intermolecular duplexes, or both types of structure at the equilibrium. Although the oligonucleotides that form either type of structure induce production of Th1-type cytokines and chemokines, including high levels of IFN-α in human PBMC and pDC cultures, only the compounds that form intermolecular duplexes induce cytokine secretion in mice [93]. In nonhuman primates, the agonists that form intermolecular duplexes induce IFN-α and IP-10 secretion. By contrast, the agonist that forms an intramolecular duplex induces only lower levels of cytokines in nonhuman primates, suggesting that this type of structure formation is less immune-stimulatory in vivo than the other structures. Consistent with these results, a TLR9 agonist containing a palindromic sequence with a potential to form an intramolecular hairpin structure induced low levels of IFN-α with no dose response at a dose range of 1–20 mg in a clinical trial in healthy subjects [94].

4.2.5 Impact of Linker on Immune Stimulation

Further, we have studied the impact of length, nature, and stereochemistry of the linker incorporated in agonists on the TLR9-mediated immune responses [95]. A number of linkers, including 1,2,3-propanetriol; (*S*)-(−)-1,2,4-butanetriol; 1,3,5-pentanetriol; *cis*,*cis*-1,3,5-cyclohexanetriol; *cis*,*trans*-1,3,5-cyclohexanetriol; 1,3,5-tris(2-hydroxyethyl)isocyanurate; tetraethyleneglycol; and hexaethyleneglycol were studied in TLR9 agonists (Fig. 5). These studies have allowed an understanding of the requirement of distance between the two 5′-ends, rigid verses flexibility of linker, and spatial arrangement of the two branches for optimal immune responses. Results from these studies suggest that C3–C5 linkers, 1,2,3-propanetriol, (*S*)-(−)-1,2,4-butanetriol, or 1,3,5-pentanetriol are optimal for stimulation of TLR9-mediated immune responses. Rigid C3 linkers with different stereochemistry have little effect on immune stimulation, whereas linkers longer than C5 reduce TLR9-mediated immune stimulation.

4.3 Design of Novel TLR9 Agonists for Therapeutic Applications

The combinations of novel structures and synthetic immune-stimulatory motifs described above have provided us with tools to generate libraries of novel synthetic agonists of TLR9, referred to as immune-modulatory oligonucleotides (IMOs). Systematic studies of several TLR9 agonists that have two 5′-ends and contain synthetic CpR dinucleotides in different nucleotide compositions in mouse, human, and monkey systems suggest that nucleotide sequence and secondary structure play a role in modulating the immune response. Based on these studies, we have broadly identified four different groups of synthetic agonists of TLR9 (Fig. 6). All four types of TLR9 agonists contain two short segments of phosphorothioate oligonucleotides with a synthetic immune-stimulatory motif (CpR, YpG, or R′pG) attached through their 3′-ends. The first two types of compounds (agonists 1 and 2; Fig. 6) do not contain a palindromic sequence; hence they do not form secondary structures. The other two types (agonists 3 and 4; Fig. 6) form intermolecular secondary structures as a result of the presence of a palindromic sequence. TLR9 agonists that contain a CpR dinucleotide motif activate TLR9, but not TLRs 3, 7, or 8, suggesting that the CpR motif is recognized by TLR9 [78, 79]. TLR9 agonists with synthetic motifs induce cell surface activation marker expression and proliferation of human B cells, but only compounds that form a secondary structure produce IFN-α in human pDC and PBMC cultures [79, 93].

All four types of compound induce cytokine production in vivo in mice and nonhuman primates. TLR9 agonists induce IFN-α in vivo in monkeys; however, the agonists that form an intermolecular secondary structure induce higher levels of IFN-α and IP-10, and activation of NK cells than those agonists without secondary

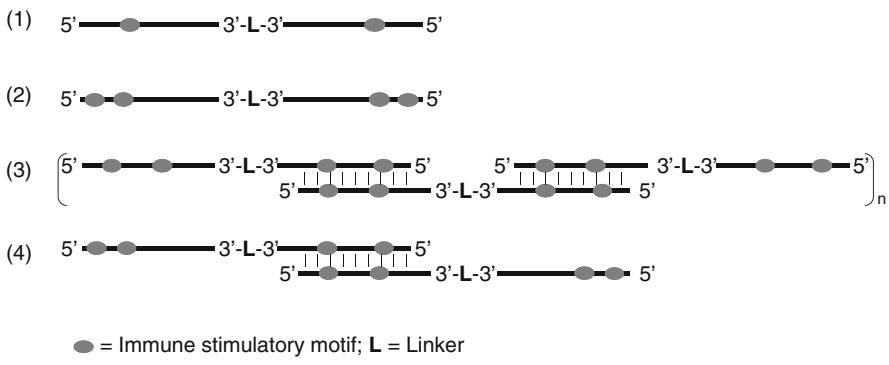

Fig. 6 Different types of TLR9 agonists. *Thick lines* represents nucleotide sequences and *oval bulges* represent synthetic immune stimulatory motifs, CpR or YpG, where R and G stand for the cytosine and guanine modifications, respectively, shown in Fig. 5. *L* represents a nonnucleotidic linker as shown in Fig. 5. Structures *1* and *2* are designed not to have palindromic sequences and hence do not form secondary structures. Structures *3* and *4* are designed with a palindromic sequence either in both branches or in one branch, respectively; hence, they can form a multimeric (*3*) or dimeric (*4*) intermolecular secondary structure as shown

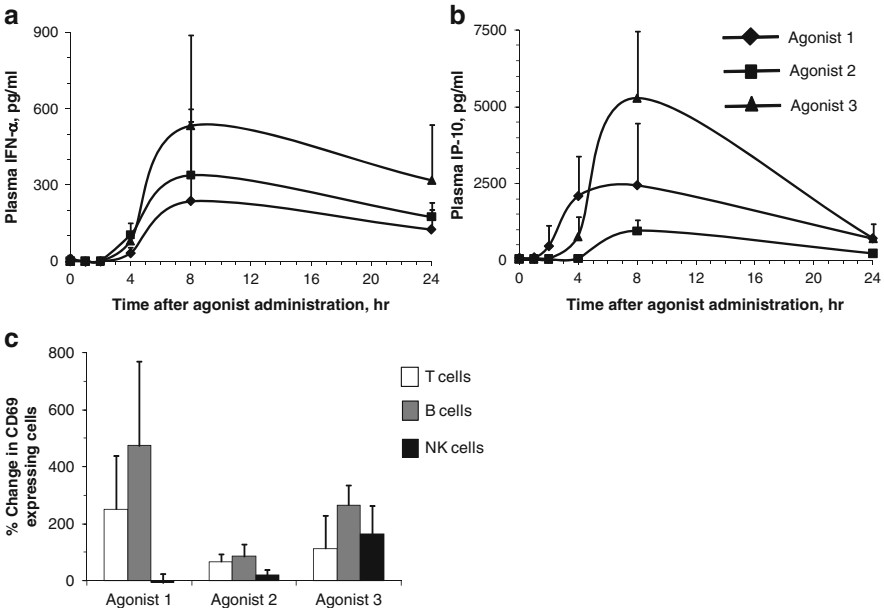

Fig. 7 Immune response profiles of representative TLR9 agonists in nonhuman primates. TLR9 agonists *1*, *2*, and *3* correspond to structures 1, 2, and 3, respectively, shown in Fig. 6. (**a**) Plasma IFN-α, (**b**) plasma IP-10, and (**c**) changes in CD69 expression on B cells, T cells, and NK cells in cynomolgus monkeys following s.c. administration of 1 mg/kg of the TLR9 agonists. Data are adopted from Kandimalla et al. [79] and Agrawal and Kandimalla [28]

structure (Fig. 7). Both in vitro and in vivo, TLR9 agonists that have two 5′-ends and contain synthetic immune-stimulatory motifs induce significantly lower levels of IL-6, but similar or higher levels of IL-12, than PS oligonucleotides containing CpG motifs [41, 76, 78, 79, 87, 90].

4.3.1 Studies of TLR9 Agonists in Various Preclinical Disease Models

A number of preclinical studies have shown that TLR9 agonists provide protection against viruses, bacteria, parasites, and fungi through the induction of Th1-type cytokines (e.g., IL-12, IFN-α, and IFN-γ [96]. TLR9 agonists also boost Th1-type acquired immune responses when used as adjuvants in combination with protein, peptide, and DNA vaccines in preclinical and clinical studies [96, 97]. Recent studies have shown the potential application of TLR9 agonists with two free 5′-ends and synthetic immune-stimulatory motifs as adjuvants with HBsAg [98], HIV gp-120-depleted whole killed HIV [99], HER-2/neu DNA vaccine [100], and TERT DNA vaccine [101, 102] in preclinical animal models.

A number of preclinical studies have explored TLR9 agonists as anticancer agents either alone or in combination with chemotherapeutic agents, and clinical trials are in progress [41, 103, 104]. Studies of TLR9 agonists in combination with EGFR and VEGF inhibitors have shown enhancement of antitumor effects via interference with the EGFR-related signaling pathways and reduction in angiogenesis in addition to TLR9-mediated immune response effects [105, 106]. In addition, a cooperative effect of TLR9 agonist plus trastuzumab has been observed in a trastuzumab-resistant breast cancer model as a result of direct antitumor and antiangiogenic activity of trastuzumab and antibody-dependent cell cytotoxicity (ADCC) enhancement by TLR9 agonist [107].

The adjuvant activity of an IMO in combination with a HER-2/neu plasmid DNA electroporation (DNA-EP)/adenovirus vaccine (vaccine) has been studied in BALB/Neu T mice [100]. Immunization of BALB/Neu T mice with a combination of vaccine and IMO results in significant tumor regression or delay to tumor progression. [^{18}F]FDG microPET and microCT imaging of mice suggest greatly reduced tumor size in the group receiving the combination of vaccine and IMO compared with the vaccine-alone group. Mice treated with the combination of vaccine and IMO produce higher antibody titers, with an IgG2a isotype switch, and have greater ADCC activity than mice treated with vaccine alone. Most importantly, the enhanced immunogenicity of the combination vaccine and IMO translates into prolonged survival of tumor-bearing mice [100].

In another mouse model of cancer, IMO has been combined with a genetic vaccine targeting murine TERT (mTERT) based on DNA electroporation (DNA-EP) and Adenovirus serotype 6 (Ad6) [101]. In this model, IMO induces dose-dependent cytokine secretion and activation of NK cells. Most importantly, vaccination of mice with IMO in combination with mTERT vaccine confers therapeutic benefits in tumor-bearing mice. Importantly, the therapeutic effect of the combination of mTERT vaccine and IMO correlates with increased NK, DC, and T-cell infiltration into the tumor microenvironment, suggesting the benefit of the combination of vaccine with IMO [101].

The efficacy of DNA-EP and Ad6 to induce immune responses against human TERT (hTERT) in nonhuman primates (*Macaca mulatta*) in combination with an IMO has been evaluated [102]. Monkeys dosed weekly with IMO concurrently with a hTERT vaccine regimen show increases in cytokine secretion and activation of NK cells compared with the monkeys that received vaccine alone. Using a peptide array, a specific profile of B-cell reactive epitopes has been identified when hTERT vaccine is combined with IMO. Although the combination of IMO with hTERT vaccine does not affect vaccine-induced TERT-specific cell-mediated immunity (CMI), this combination elicits multiple effects on innate and adaptive immune responses in nonhuman primates, including TERT-specific antibody production, IgG2a isotype switch, and ADCC [102].

TLR9 agonists have been shown to prevent and reverse allergen-induced Th2 immune responses that are commonly observed in asthma and allergic conditions in a number of preclinical models [108], and clinical studies are underway [109]. In ovalbumin (OVA)-sensitized mouse models of asthma, agonists of TLR9

containing novel structures and synthetic immune-stimulatory motifs prevent the development of allergic airway inflammation and airway hyper-responsiveness when coadministered during OVA-sensitization [110] and reverse established allergic responses when administered to OVA-sensitized and – challenged mice [111]. Novel TLR9 agonists can also be used to prevent and treat OVA-induced Eustachian tube dysfunction in rat models, suggesting that they might be useful in treating allergy-associated otitis media with effusion (OME), a chronic inflammatory condition of the middle ear [112–114].

4.3.2 Clinical Studies on TLR9 Agonists

Three TLR9 agonists that induce distinct immune response profiles based on their structures have been selected for clinical studies. IMO-2055 is a lead clinical candidate for the treatment of cancers. The safety and pharmacodynamic activity of IMO-2055 have been studied in Phase-I rising-dose trials in healthy and cancer subjects. A dose-dependent transient migration of lymphocytes from peripheral blood, activation of surface markers on immune cells, and elevation of plasma cytokines has been observed, consistent with immune-stimulatory activity of IMO-2055. No significant acute phase or proinflammatory activity, including no effects on cortisol, C-reactive protein (CRP), and erythrocyte sedimentation rate (ESR), were observed [41, 115–118].

A Phase-1 safety study of IMO-2055 in combination with gemcitabine and carboplatin in advanced, previously treated solid tumor cancer patients has been reported [119]. Patients in the study received 3-week cycles of fixed-dose gemcitabine and carboplatin with three escalating doses of IMO-2055. The dose-limiting toxicities in these patients include neutropenia and thrombocytopenia, which are common side effects observed with gemcitabine and carboplatin. In this study, the response rate, progression-free survival (PFS), and overall survival rates were 5%, 4.1 and 12.9 months, respectively. In a subset of eight patients with nonsmall cell lung cancer (NSCLC), the response rate, PFS, and overall survival rates were 13%, 6.5 and 12.9 months, respectively [119].

A Phase-2 clinical study of IMO-2055 monotherapy in renal cell carcinoma with four arms has been reported [120]. In this study treatment, naïve and second-line patients received IMO-2055 subcutaneously at either 0.16 or 0.64 mg/kg/week. The median PFS was 4.5 and 1.9 months for the 0.16- and 0.64-mg/kg/week treatment-naïve patients, and 3.4 and 4.3 months for the 0.16- and 0.64-mg/kg/week second-line patients, respectively. Median overall survival was 23.5 months over all arms, with 58% of patients having stable disease. Two patients have shown partial responses. Seven patients received weekly IMO-2055 treatment for at least 1 year. IMO-2055 treatment was generally well tolerated at the dose levels studied. The primary objective of tumor response, based on response evaluation criteria in solid tumors (RECIST), was not achieved in the study [120].

IMO-2055 has been studied in a Phase-1b clinical study in combination with Avastin and Tarceva in patients with NSCLC [121]. In this study, IMO-2055 was

administered at four escalating dose levels up to 0.48 mg/kg/week with fixed standard dose regimens of Avastin and Tarceva. IMO-2055 is well tolerated at all dose levels, and eight of the 16 patients remain on treatment for at least 18 weeks. Of the 13 patients evaluable for tumor response, three have shown a partial response and eight experienced stable disease [121]. Based on this study, a dose level of IMO-2055 has been selected for expanded patient recruitment to evaluate further the safety and pharmacokinetics of the combination.

A Phase-1b clinical trial of IMO-2055 in combination with Erbitux (a recombinant, humanized antibody to EGFR) and chemotherapy in patients with colorectal cancer, whose cancer has progressed during a prior course of standard therapy, is now underway. Additionally, a Phase-2 clinical trial of IMO-2055 in combination with Erbitux in second-line Erbitux-naïve patients with recurrent or metastatic squamous cell carcinoma of the head and neck is underway.

A second candidate, referred to as IMO-2134, induces potent Th1-type immune responses in vitro in human cell-based assays and downregulates allergen-induced Th2-type responses in mice. In a nonhuman primate model of *Ascaris suum* challenge, administration of IMO-2134 by aerosol inhalation effectively attenuates inflammatory responses in the lungs after repeated allergen challenges. IMO-2134 inhibits IL-13 in (bronchoalveolar lavage) BAL fluid after the allergen challenge, increases IP-10 levels in plasma, and induces expression of several IFN-dependent genes in BAL cells, indicating inhibition of Th2 responses and induction of a Th1-like immune response [122]. A Phase-1 clinical study of IMO-2134 has been carried out following intranasal delivery.

A third candidate, referred to as IMO-2125, is a representative member of the compounds (agonist 3 in Fig. 6) that induce high and sustained levels of IFN-α and activation of NK cells in nonhuman primates. IMO-2125 has been selected as a candidate for the treatment of Hepatitis C, where recombinant IFN-α in combination with ribavirin is the standard of care. IMO-2125 is currently being evaluated in Phase-1 trials in hepatitis C patients alone and in combination with ribavirin.

Interim data from an ongoing phase 1 trial in chronic Hepatitis C patients who did not respond to previous treatment with standard of care (null-responders; who failed to achieve a 2log10 reduction in viral load during a previous 12–24 week treatment with PEGylated-recombinant IFN-α and ribavirin) has been reported [123]. Genotype 1a or 1b nonresponder patients with elevated plasma IP-10 levels were treated with IMO-2125 subcutaneously once a week for 4 weeks at dose levels of 0.04, 0.08, 0.16, or 0.32 mg/kg. No drug-related discontinuations or serious adverse events (AEs) have been reported. Most common AEs include flu-like symptoms, injection site pruritus, fatigue, headache, arthralgia, and myalgia. Dose-dependent increases in plasma IP-10, IFN-α, and 2′5′-oligoadenylate synthetase at 24 h after IMO administration have been reported. The induction of IFN-α is stronger after the fourth dose compared with the first dose. Of the eight null-responder HCV patients that received 0.32 mg/kg/week of IMO-2125, six showed maximum viral load reductions ranging from 1.0 to 3.5log10 at least once during the treatment period [123]. The levels of plasma IFN-α induced by IMO-2125 correlates with viral load reduction. Enrolment is continuing at a 0.48 mg/kg/week dose level of IMO-2125.

5 TLRs and Autoimmune Diseases

When self-tolerance breaks down, the immune system recognizes self-antigens and produces inflammatory responses and antibodies that attack the body's own tissues, leading to autoimmune diseases. Immune complexes containing self-nucleic acids are shown to act as endogenous ligands for TLR7 and TLR9, stimulating IFN-α and other proinflammatory cytokines and exacerbating autoimmune diseases [124–126]. TLR-mediated IFN-α induction by endogenous ligands has been implicated in several autoimmune and inflammatory diseases such as lupus, psoriasis, and atherosclerosis [126–129] (Fig. 8).

Monoclonal antibodies targeting a variety of downstream elements, such as TNF-α, IL-12, IP-10, IFN-α, and BLyS, are currently being developed or are in use for the treatment of autoimmune diseases, including rheumatoid arthritis (RA), lupus, and psoriasis. Hydroxychloroquine, an inhibitor of endosomal TLR-mediated immune responses via neutralization of endosomal acidity, is a commonly used drug for the treatment of lupus, RA, and psoriasis [130]. Therefore, compounds that act as antagonists of TLR7 and TLR9 by suppressing the immune

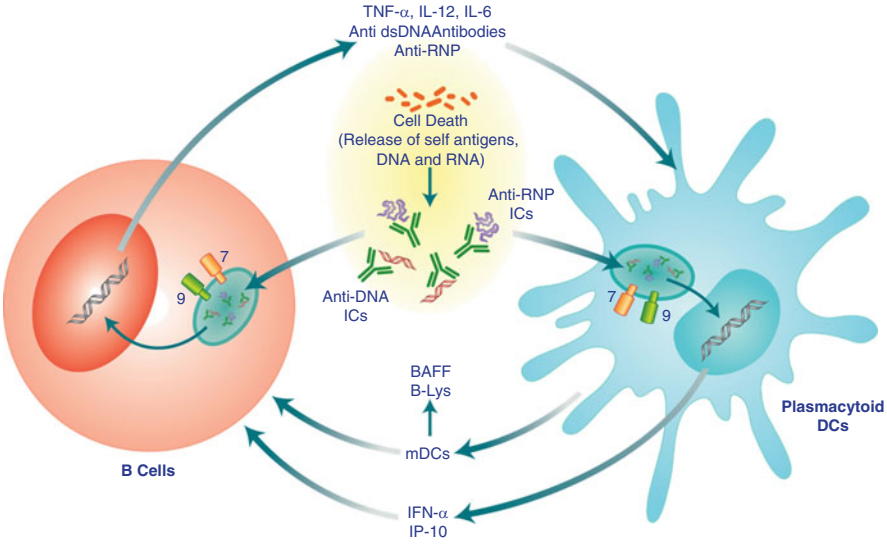

Fig. 8 Role of TLR-mediated inflammation in autoimmune diseases. Immune complexes (*ICs*) containing apoptotic and necrotic cell-derived nucleic acid materials (*center*) are taken up into endolysomes of pDCs via FcγRIIa-mediated uptake (*right*) and into B cells through engagement of B cell receptors (*left*). Engagement of TLR7 (*7*) and TLR9 (*9*) by immune complexes containing self-nucleic acid leads to induction of type I interferons (*IFN-α*) and other proinflammatory cytokines in pDCs, and stimulation of proliferation, induction of cytokines, and production of autoantibiodies by B cells. The uptake of self-nucleic acid–autoantibody complexes can be facilitated by molecules such as high mobility group box 1 protein (HMGB1), which is released from necrotic cells, and LL37, an antimicrobial peptide released from injured skin cells. *RNP* ribonuclear protein, *BAFF* B cell activating factor, B-Lys B lymphocyte stimulator

responses mediated through these receptors would have wide application in the treatment of autoimmune and inflammatory diseases.

5.1 Antagonists of TLR7 and TLR9

Certain synthetic oligodeoxynucleotides containing guanine-rich sequences along with sequence motifs other than CpG act as antagonists and inhibit TLR9 activation by CpG oligonucleotides [131–137]. The mechanism of action of guanine-rich oligonucleotide antagonists of TLR9 is not well understood, but the evidence suggests that they may interfere with STAT1, STAT-3, and STAT-4 and/or other downstream factors proximal to NF-κB activation involved in the signaling pathways of TLR9 [137, 138]. These types of oligonucleotides, depending on the nucleotide sequence, also act as agonists of TLR9 [51, 52]. Guanine-rich oligonucleotides interact with a variety of protein factors and receptors of cells and have antitumor and anti-infective activities [139–142].

We have evaluated the secondary structure formation by some of the guanine-rich oligonucleotides that have been reported as TLR9 antagonists by nondenaturing polyacrylamide gels and UV thermal melting studies. As shown in Fig. 9, all of the compounds studied form a secondary structure as determined by measurement of melting temperature (T_m) and change in absorbance with thermal dissociation (% hyperchromicity) (Fig. 9a). Consistent with UV thermal melting results, several compounds show slower (multistranded structures) and/or faster (hairpin-type structures) mobility bands on nondenaturing gel (Fig. 9b). These results clearly demonstrate that guanine-rich oligonucleotides form hyperstructures in vitro and possibly in vivo. Guanine-rich oligonucleotides lack pharmaceutical attributes due to unpredictable secondary-structure formation, depending on the experimental conditions and nonsequence-specific protein binding, including immune stimulation [147, 148].

The potential of oligonucleotides containing an unmethylated CpG motif, in which two nucleotides adjacent to the CpG dinucleotide are replaced with 2′-*O*-methylribonucleotides to act as TLR7 and TLR9 antagonists has been reported recently [149]. In mouse and human cell cultures, these antagonists do not stimulate immune activation, but inhibit TLR7 and TLR9 agonist-induced activity. In mice, antagonists inhibit immune responses induced by TLR9 agonists for up to 5 days, and the inhibition is dose-dependent [149]. Antagonists also inhibit immune responses induced by an RNA-based TLR7 or TLR8 agonist, but not those induced by agonists of TLRs 2, 3, 4, or 5, in mice [149] (Fig. 10). Additionally, such an antagonist inhibits TLR9-agonist-induced IL-6 in lupus-prone MRL/lpr mouse spleen cell cultures [149]. These results indicate that antagonists with the type of structures described can suppress both TLR7- and TLR9-mediated immune responses.

We have also studied the immune-stimulatory and antagonistic activity of CpG-containing ODNs in which cytosine or guanine was replaced with 2′-*O*-methylribonucleotides, 5-methyl-dC, or 2′-*O*-methyl-5-methyl-C [150]. In mouse

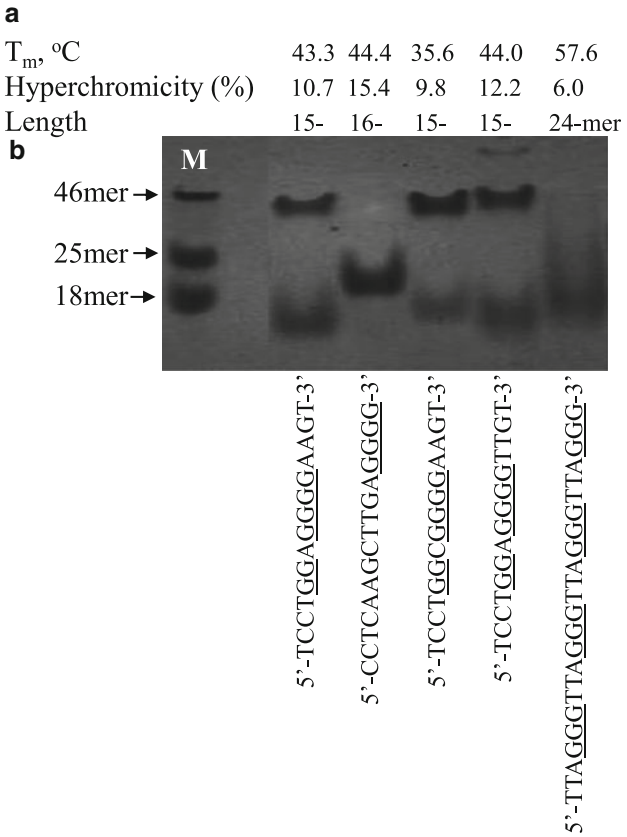

Fig. 9 (**a**) Thermal melting and (**b**) gel-mobility shift data for guanine-rich oligonucleotides that have been reported as TLR9 antagonists. Guanine-rich oligodexynucleotides form secondary structures that show distinct thermal melting temperatures (T_m) in UV thermal melting studies. They also form hairpin or multistranded structures as observed with different mobilities on nondenaturing polyacrylamide gel electrophoresis. Sequences (from *left* to *right*) are taken from Patole et al. [143], Yamada et al. [144], Stunz et al. [145], Barrat et al. [132], and Dong et al. [146]

and human primary cell-based assays, modified ODNs do not stimulate immune responses, but do inhibit TLR9 agonist-induced immune-stimulatory activity. Similarly in mice, modified ODNs do not induce cytokines, but they do inhibit immune responses induced by agonists of TLR7 and TLR9 [150]. By contrast, modified ODNs do not inhibit endosomal TLR3- or cell-surface TLR4-agonist-induced cytokines [96]. Results of these studies demonstrate that ODNs that have chemical modifications in CpG dinucleotides do not induce immune-stimulatory activity but act as antagonists of TLR7 and TLR9 in vitro and in vivo [150]. These types of modifications are commonly employed in antisense sequences, and thereby may affect the intended antisense mechanism of action.

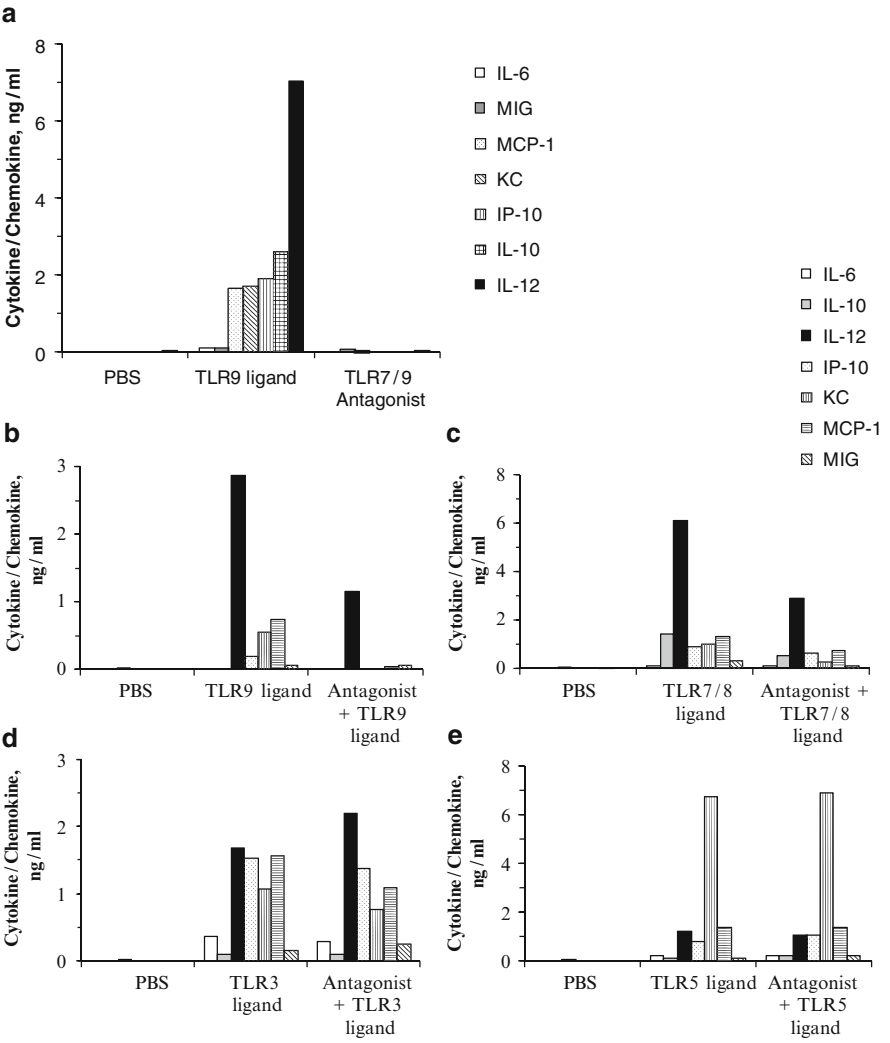

Fig. 10 Activity of dual TLR7/9 antagonist in mice. (**a**) Serum cytokine profiles of a TLR9 ligand and a dual TLR7/9 antagonist (20 mg/kg) in C57BL/6 mice. C57BL/6 mice were injected s.c. with agonist (0.25 mg/kg), antagonist (20 mg/kg), or phosphate-buffered saline (*PBS*). Blood was collected 2 h after compound administration, and serum analyzed for cytokine levels by luminex bead assay. (**b**–**e**) Specificity of inhibition of TLR agonist-induced immune responses by antagonist in mice. C57BL/6 mice were injected s.c. with antagonist (10 mg/kg) in the right flank. After 72 h, (**b**) TLR9 ligand (0.25 mg/kg), (**c**) TLR7/8 ligand (50 mg/kg), (**d**) TLR3 ligand (polyI.polyC, 20 mg/kg), or (**e**) TLR5 ligand (flagellin, 0.25 mg/kg) were injected s.c. in the left flank. Two hours later blood was collected, and serum levels of cytokines were determined by luminex bead assay. *KC* keratinocyte-derived chemokine, *MCP-1* macrophage chemotactic protein 1, *MIG* monokine induced by interferon-γ. Data are adopted from Wang et al. [149]

5.2 Therapeutic Applications of Antagonists of TLR7 and TLR9

Antagonists may be suitable candidates for treating inflammatory and autoimmune diseases, where inappropriate or uncontrolled TLR activation has been implicated. An antagonist candidate, referred to as IMO-3100, has been selected as a lead candidate for clinical evaluation in autoimmune and inflammatory diseases such as lupus, rheumatoid arthritis, psoriasis, and colitis.

6 Conclusions

Synthetic oligonucleotides comprising various nucleotide sequences, lengths, and chemical modifications are currently being studied as therapeutic agents based on a broad range of mechanisms of action, including mechanisms involving antisense, ribozymes, siRNA, aptamers, and immune modulators. Following uptake, oligonucleotides are commonly transported into intracellular compartments by endocytosis. TLRs 3, 7, 8, and 9, which recognize nucleic acid-associated molecular patterns, are expressed in the endosomes. Through extensive structure–activity relationship studies, we have gained insights into how chemical and structural modifications of oligonucleotides affect their interaction with endosomal TLRs. Due considerations should be given in selecting sequences and modifications of oligonucleotides to make sure that the mechanisms by which they are acting are intended. As described, we have created oligonucleotides that act as agonists of TLRs 7, 8, or 9 or antagonists of TLR7 and TLR9. These classes of compounds have very broad therapeutic applications and are currently being studied in clinical trials.

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Delivery of Nucleic Acid Drugs

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Abstract Nucleic acid drugs should be delivered into the corresponding intracellular target site, i.e. the nucleus or cytosol. Because biosystems have developed several barriers to prevent the intrusion of external genetic material, highly sophisticated delivery machinery must be used to overcome those barriers. In this review, we describe the evolution of polymer-based carriers over several decades of research. To prevent deactivation in extracellular circumstances, nucleic acid drugs are condensed with cationic polymers or encapsulated with hydrophobic polymers. The condensed or encapsulated complexes are often crosslinked to enhance the physicochemical stability and coated with biocompatible polymers for safety. Delivery of the nucleic acid drugs to a specific site and subsequent intracellular entry can be partially accomplished by passive targeting based on the enhanced permeability and retention (EPR) effect or nonspecific endocytosis, but more effectively by receptor-mediated endocytosis. After the cell entry, various functionalities help the nucleic acid drugs escape the endosomes to enter the cytosol, release them from the carrier system, and transfer them into nucleoplasm through the nuclear envelope. The development of an ideal delivery carrier with efficiency, specificity, as well as safety has been supported by progress in polymer

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chemistry and supramolecular chemistry, and accumulated experience in biological and clinical trials.

Keywords Biocompatibility · Biosignal sensitivity · Gene delivery · Nonviral carriers · Oligodeoxynucleotide (ODN) delivery · Polyion complex (PIC) micelle · Polymeric carriers · siRNA delivery

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1 Introduction

The concept of drug delivery has been applied to almost all types of drugs for the improvement of efficiency, safety, and specificity. The efficiency, safety, and lack of side effects of drugs are important goals in the field of pharmacy and medicinal chemistry. However, the selection of an appropriate drug delivery technique can provide completely new potential for a pre-existing drug by remarkably improving its therapeutic effects. In contrast to low molecular weight hydrophobic drugs, which can freely pass through the plasma membrane, most nucleic acid drugs have difficulty penetrating to the target site, cytoplasm, or nucleoplasm due to their ionic characteristics and high molecular weight. Therefore, the careful selection of an optimal delivery system according to the function and target site is essential for therapeutics using nucleic acid drugs.

Viral carriers have been recognized as the most efficient system for nucleic acid delivery, especially in the case of DNA. Through the course of evolution, viruses such as adenovirus [1], adeno-associated virus (AAV) [2], and retrovirus [3] have developed very sophisticated mechanisms for the delivery of their genomes into host cells. The viral machinery can be used as an efficient vehicle for gene delivery, if the expression of the original viral genes can be prevented by the exchange of almost all infectious viral genes with therapeutic genes. Molecular biologists have

developed genetically modified viral carriers by careful recombinant techniques, and it has been assumed that the dangerous wild-type viruses cannot be reproduced after the delivery of the therapeutic gene.

However, it has been reported that even modified viral carriers can induce excessive immune response because they still contain viral capsid proteins on the surface. The clinical trial of one adenoviral gene therapy ended in the catastrophic death of a patient due to a multiorgan collapse from excessive innate immune response; this tragic event has caused doctors to reconsider the clinical use of viral carriers [4]. In another case, leukemia was generated during retroviral gene therapy because the retrovirus randomly integrated the genetic materials into the host chromosome during the transfection process [5]. Other weaknesses of viral carriers such as the complexity of the production procedure, the difficulty of large-scale production, and the possible reproduction of the wild-type virus stand as roadblocks to their development.

Therefore, nonviral delivery carriers are now being highlighted as alternatives to viral carriers [6]. To mimic the viral carrier system, various supramolecular assemblies based on lipids [7], polymers [8], and inorganic materials [9] have been developed, although nonviral technologies also include physical methods such as naked DNA injection [10], use of a gene gun [11], electroporation [12], and sonication [13]. The clinical use of nonviral carriers has rapidly increased due to their strong points such as low immune response, easy large-scale production, and temporary gene expression without integration into the host chromosome. Although nonviral vectors have lower efficacy of gene transfection compared with viral vectors, they may have a higher maximum tolerant dose (MTD), resulting in appreciable in vivo efficacy.

Lipids and polymers are the most frequently used materials among nonviral carriers. The lipid molecules form supramolecular structures such as liposomes or vesicles, for which the main driving force is intermolecular hydrophobic interaction in an aqueous environment. Because most of the nucleic acid drugs have negative charges due to the phosphate backbone, the electrostatic interaction between the cationic lipid molecules and the nucleic acid drugs is also a key factor for the formation of the supramolecular complexes. The lipid molecule has a relatively simple structure that can be easily synthesized; however, it is difficult to introduce signal-responsive functional groups or specific ligands to such lipid-based carriers due to this very simplicity of their structure.

By contrast, the relatively large and complex structure of polymer-based carriers provides a high potential for various functionalities to be introduced. Although the main strategy for the encapsulation of nucleic acid drugs was electrostatic complexation by cationic polymers, other strategies used were covalent bond conjugation [14], emulsifying encapsulation [15], and hydrogel formation [16]. For the controlled release of the nucleic acid drugs, chemical bonds responding to pH [17], temperature [18], and reduction potential [19] have been introduced in many polymer-based carriers. Of course, knowledge from the lipid-based carriers [20], inorganic delivery materials [21], or even physical delivery methods [22] is now being applied to the design of polymer-based carriers.

This review is focused on the design and application of polymer-based carriers for the delivery of nucleic acid drugs. After a short discussion of the biological

barriers preventing the delivery of nucleic acid drugs, strategies for improving polymeric delivery systems will be described in detail, starting from the basic polymeric materials. Smart polymers responding to biosignals, biodegradable polymers, and ligand introduction are at the center of improvements to the efficiency, safety, and specificity, respectively.

2 Biological Barriers in the Delivery of Nucleic Acid Drugs

2.1 Extracellular Barriers

Most nucleic acid drugs are biologically active only after their uptake into cells, and the site of the function varies with the drug type. Mononucleotide drugs prevent the replication of chromosomal DNA, and plasmid DNA (pDNA) should be transcribed in the nucleus. Antisense DNA (asDNA) and small interference RNA (siRNA), the targets of which are the corresponding mRNA, show their function in cytosol. To access the final target site, the nucleic acid drugs must overcome several barriers that the biosystem has developed for protection from invasion by external microorganisms. These biological barriers can be classified into extracellular and intracellular barriers.

After the injection of nucleic acid drugs into the blood stream, most of the low molecular weight drugs are rapidly excreted by the kidney because the cut-off value of the glomerular filtration is around 20,000 Da (Fig. 1) [23]. Because the delivery efficiency of drugs is dependent upon the concentration in the blood stream, it is difficult to enhance the delivery efficiency unless the molecular weight of the delivery

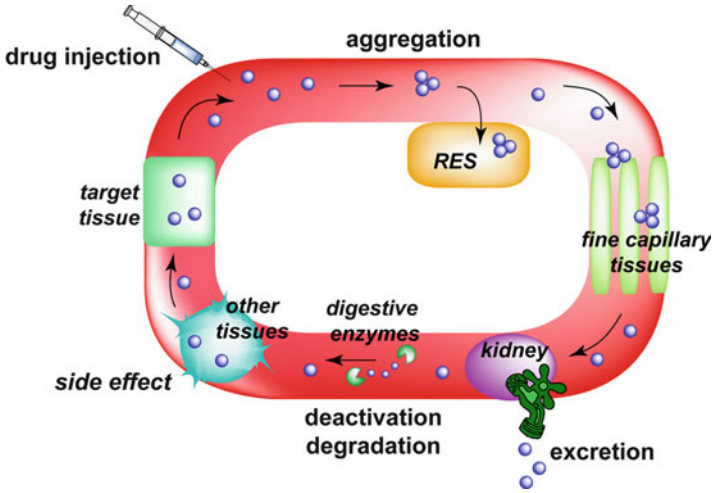

Fig. 1 Extracellular barriers to nucleic acid drug delivery

vehicle is over the cut-off value. In many cases, the molecular weight could be increased by complex formation, encapsulation, or conjugation with various polymers. Nucleic acid drugs can also be protected from degradation or digestion by the extracellular nucleases through the formation of supramolecular structures [24].

However, the increase in the molecular weight or the formation of supramolecular structures creates other problems. Colloidal particles with a diameter of over 300 nm were rapidly trapped by the reticuloendothelial system (RES) in the liver, spleen, and lung before they reached the target tissue [25]. Even particles with an initial diameter of less than 300 nm can form large aggregates by the interaction with serum proteins such as albumin, immunoglobulins, and complements [26]. Because most serum proteins have net negative charges, cationic polymer-based carriers have a strong tendency to form aggregates with serum. The aggregates accumulate in organs with fine capillary structures such as the lung, skin, or intestine and can block the blood stream [27]. On the other hand, a strongly negatively charged carrier system can be removed from the blood stream by non-parenchymal cells such as Kupffer cells in the liver [28]. Therefore, careful control of the surface charge and size of carriers is essential in overcoming extracellular barriers efficiently.

2.2 Cell Membrane and Endocytosis

The plasma membrane itself, which compartmentalizes the extracellular matrix and the intracellular cytosol, is the second barrier against nucleic acid drugs after they overcome the extracellular barriers. Although hydrophobic small molecules can diffuse freely into the cytosol, charged molecules have difficulty penetrating through the plasma membrane, which is composed of a lipid bilayer. Moreover, the highly negatively charged nucleic acid drugs have even more difficulty interacting with the plasma membrane, which has an extracellular surface with the same negative charges due to the high content of anionic glycosylated membrane proteins [29]. Therefore, the nucleic acid drugs must use specialized pathways into the cytosol – pathways initially developed for the internalization of extracellular nutrients and signal molecules. Most nonviral carriers as well as viral carriers can be internalized into cells via the endocytosis, phagocytosis, or macropinocytosis pathways [30].

Relatively large particles with a diameter of 1–10 μm are internalized by phagocytosis or macropinocytosis. Phagocytosis is the mechanism of clearing large pathogens or cell debris, primarily conducted by macrophages. Macropinocytosis, which engulfs large volumes of extracellular fluid, is an important pathway for antigen uptake in dendritic cells (DC) or antigen-presenting cells (APCs). Hence, the induction of phagocytosis or macropinocytosis using microsized particles can be a useful strategy in the application of nucleic acid drugs for immunization, as in the case of a DNA vaccine [31].

Endocytosis is well-developed in most cell types, and is classified into receptor-mediated endocytosis and nonspecific endocytosis on the basis of ligand–receptor

interaction. Various ligands induce the receptor-mediated endocytosis, which can enhance the specificity of the delivery into target cells. On the other hand, nonspecific endocytosis mediates the internalization of nucleic acid drug carriers without any ligand. In many cases, nonspecific endocytosis was induced by the electrostatic interaction between the delivery carriers and proteoglycans on the surface of the plasma membrane [32]. Cationic carriers can interact more effectively with proteoglycans having negatively charged sulfate or carboxylate groups, so that they generally show higher endocytic efficiency than anionic carriers, although some controversial results have been reported [33].

Depending on the mechanism, endocytosis can also be classified into clathrin-mediated endocytosis [34], caveolin-mediated endocytosis [35], and clathrin- and caveolin-independent endocytosis [36]. Clathrin-mediated endocytosis is a major entry mechanism, and also the most intensely investigated mechanism among the various forms of endocytosis. Although clathrin molecules are involved in most receptor-mediated endocytosis and nonspecific endocytosis, the detailed endocytic mechanism of nonviral carriers has not been well understood yet and has been shown to vary with the size, surface charges, and other characteristics of the carriers. Many carriers use several mechanisms simultaneously, probably due to the intrinsic heterogeneity of the supramolecular structure [37].

2.3 Intracellular Barriers

2.3.1 Endosomal Membrane

A vesicular organelle called an endosome is generated after the endocytosis of foreign molecules, especially in the case of clathrin-mediated endocytosis (Fig. 2). The endosome is gradually acidified by ATPases in the endosomal membrane that pump protons into the endosomal lumen from the cytosol [38]. The acidification proceeds from the normal physiological pH value of 7.4 to the lysosomal pH value of 4.8. The fully acidified endosome will fuse with a lysosome, which contains hydrolytic enzymes, to degrade internal macromolecules. Therefore, for the successful delivery of intact nucleic acid drugs, it is essential to escape the endosome at the early stage of acidification prior to degradation or deactivation. Endosomal escape is a major bottleneck step in the delivery of asDNA and siRNA to function in the cytosol.

Various polymer-based carriers for nucleic acid delivery include a moiety to disrupt the endosomal membrane in a pH-dependent manner. Polymers with protonatable amine moieties with a pK_a value of 5–6 can rupture the endosomal membrane, probably as a result of endosome buffering and osmotic swelling [39]. Direct contact of the polymer-based carriers with the endosomal membrane [40] and the pH-sensitive α-helix formation of a specific peptide [41] also induced endosomolysis. The introduction of various moieties for efficient endosomal escape will be discussed in detail.

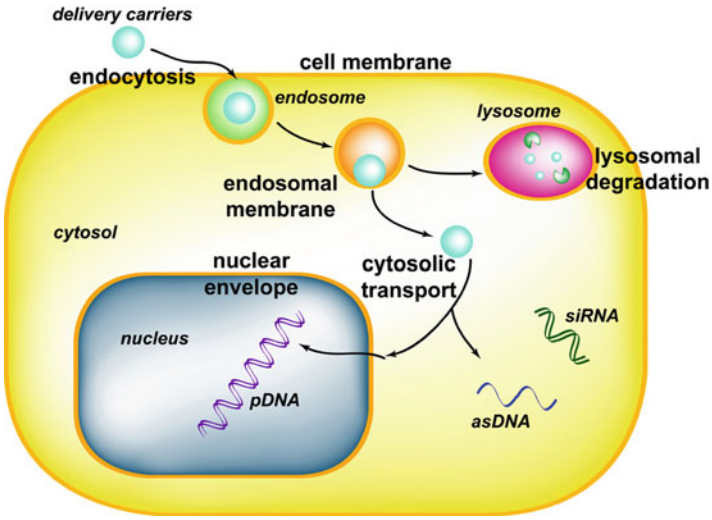

Fig. 2 Cell entry and intracellular barriers to nucleic acid drug delivery

2.3.2 Cytosolic Transport

Although asDNA and siRNA finish their journey at the cytosol just after endosomal escape, other nucleic acid drugs such as pDNA must be delivered through the cytosol into the nucleus for the transcription of the therapeutic gene. Cytosol is not a simple liquid phase that enables free diffusion of macromolecules, but a gel-like phase with a fine mesh structure primarily composed of actin filaments [42]. The diffusion rate of large molecules over a hydrodynamic diameter of 85 nm is significantly lower than that of small molecules in cytosol due to the molecular exclusion effect [43]. In addition, the cytosolic concentration of free DNA was shown to rapidly decrease, with a half-life of 90 min, by the action of nucleases preventing the invasion of viral DNA or RNA [44]. Hence, protection of pDNA from nuclease attack is needed for delivery to the nucleus without loss of activity.

In fact, nonviral carriers were reported to use some active transport mechanisms through the cytosol, although the details of these mechanisms are not well understood [45]. It was suggested that they use a similar transport mechanism as viruses, which are transported by microtubule filaments. By the active transport mechanism, the carrier can be delivered near the nuclear envelope within 30 min.

2.3.3 Nuclear Envelope

The final stage in nucleic acid drug delivery is penetration through the nuclear envelope. Along with the plasma membrane and the endosomal membrane

mentioned earlier, the double bilayer structure of the nuclear envelope is one of the main barriers to pDNA delivery. Although the nuclear pore complex (NPC) controls the transport of macromolecules between the cytosol and nucleoplasm [46], the passive diffusion cut-off value of the NPC is about 9 nm, so that it is impossible for large macromolecules such as free pDNA or polymer–pDNA complexes to enter the nucleus by passive diffusion through the NPC. The macromolecules can, however, enter into the nucleoplasm during mitosis, when the nuclear envelope disintegrates [47]. Of course, this clinical application is highly limited because most cells in the body are postmitotic.

Based on the fact that the open state of the NPC facilitates the active transport of particles less than 26 nm [48], successful delivery of pDNA into the nucleus was accomplished by the conjugation of the nucleus localization signal (NLS), which binds to importin, an important protein controlling the nuclear transport of nuclear proteins [49]. Other strategies for overcoming the hurdle of nuclear entry using a carbohydrate-binding receptor, lectin [50], or direct disruption of the nuclear envelope [51] have been intensely investigated.

3 Basic Polymers for Nucleic Acid Delivery

3.1 Poly(L-lysine)

Thousands of polymers have been developed for the delivery of nucleic acid drugs. Although some polymer-based carriers have introduced totally different concepts into the design of their backbones, most of the polymer-based carriers rely on a few basic polymers. In this section, we will describe three basic polymers, poly (L-lysine) (PLL), poly(ethylenimine) (PEI), and poly(amidoamine) (PAMAM) dendrimer in order to survey the standard concepts of polymer-based carriers.

After the compaction of chromosomal DNA by histone, proteins with high lysine contents was observed; lysine-based polymers have been used to condense nucleic acid drugs efficiently. Because the pK_a value of the ε-amine in the lysine is around 10.8, the resulting positive charges generate an electrostatic attractive force with the negative charges of phosphate groups in nucleic acid drugs at neutral pH. Hence, a peptide polymer composed of L-lysine, PLL, can form a polyion complex (PIC) with nucleic acid drugs to prevent the degradation of the nucleic acids by external nuclease attack [52].

The most common way to synthesize PLL is the ring-opening polymerization of an ε-amine-protected L-lysine *N*-carboxy-anhydride (NCA) (Fig. 3) [53]. The activated monomer can be polymerized into a protected PLL polymer by the initiation of a nucleophilic initiator. PLL is finally obtained by the deprotection of the protecting groups on ε-amines. A short alkyl amine is often selected as the initiator for the synthesis of a PLL homopolymer, but a macroinitiator has also been used for the synthesis of a PLL block copolymer or other derivatives [54]. The

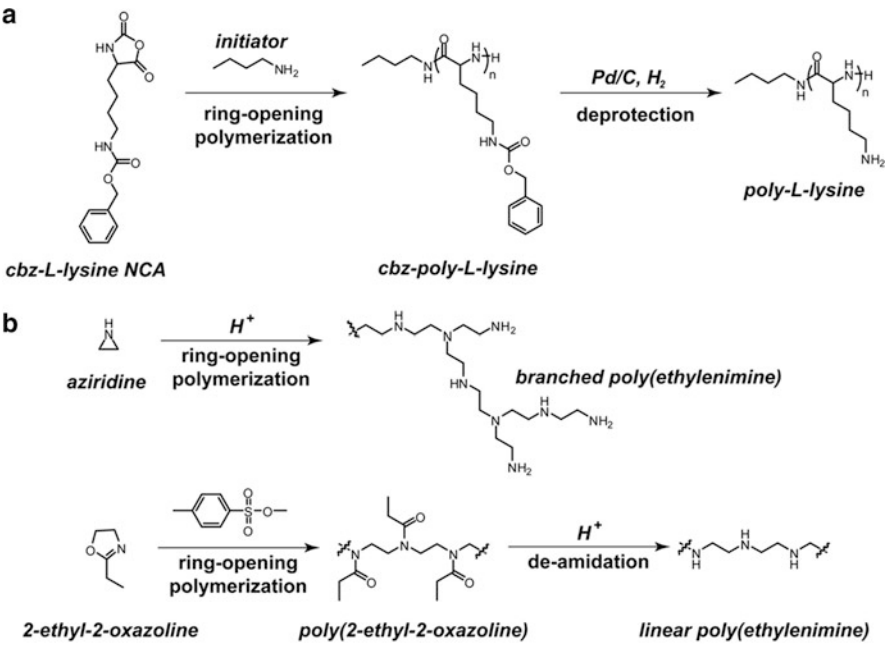

Fig. 3 Synthetic schemes for (**a**) poly-L-lysine and (**b**) branched and linear poly(ethylenimine)

molecular weight or the degree of polymerization (DP) of the synthesized PLL polymer is dependent upon the monomer-to-initiator ratio.

The molecular weight of PLL, the mixing ratio between PLL and the nucleic acid drugs, and external salt conditions all influence the stability of the PIC structure. A short PLL with a molecular weight of less than 3,000 cannot provide enough stability to form a PIC with pDNA. The amine (N)-to-phosphate (P) ratio or positive-to-negative charge ratio is generally used as a measure of the mixing ratio between cationic polymers and anionic nucleic acid drugs in most PICs. At the N:P ratio of 1, the PIC structure severely aggregates due to the charge neutralization and the resulting loss of electrostatic repulsion between the colloidal particles. The PIC between PLL and pDNA showed a toroid or rod structure with a diameter of around 50 nm at N:P ratio greater than 1, which was stabilized by the repulsive force between the similarly positive charges [55]. The PIC structure of PLL was relatively stable in water; however, incubation in physiological saline induced the aggregation of the PIC [56]. Therefore, a special PLL derivative is required to obtain a physiologically stable PIC for in vivo use [54].

The condensation of nucleic acid drugs by PLL reduces the hydrodynamic radius of nucleic acid drugs to the appropriate scale for endocytosis, and the neutralization of the anionic charges of nucleic acid drugs reduces the electrostatic repulsion against the plasma membrane. These characteristics of the PLL complex, along with the protection from external nuclease attack, significantly enhanced delivery efficiency compared to the free nucleic acid drugs. The highest delivery efficiency

was shown at the N:P ratio of 2 [57]. Complexation at lower N:P ratios may cause premature dissociation of the nucleic acid drugs, whereas inefficient unpacking of nucleic acid drugs from the complex could reduce the bioactivity of the nucleic acid drugs when the complex was formulated using a PLL with too-high a molecular weight or at too-high an N:P ratio. [58].

Although it is generally accepted that nonviral carriers are more biocompatible than viral carriers, nonviral carriers often provoke cytotoxicity. Detailed mechanisms of this induction of cytotoxicity have not been well investigated. Probably, the high density of the positive charges in cationic polymers can disrupt the plasma membrane to induce apoptotic cell death [59]. The cytotoxicity of PLL is relatively high among polymer-based carriers. In an in vitro transfection experiment with the PLL–DNA complex, the cell viability was reduced to 40–60%, especially in the case of high molecular weight PLL [60].

The simple structure of PLL, with its ability to condense anionic macromolecules, shows the basic concept of polymeric carriers for nucleic acid delivery. Various modification methods including a complete change of the backbone have been developed to improve the low delivery efficiency and reduce the high cytotoxicity of PLL. A hydrophobic moiety [61], pH-responding moiety [62], and NLS [63] have been introduced to enhance the efficiency of PLL and poly(ethyleneglycol) (PEG) [24, 60]. A biodegradable backbone [64] has also been introduced for higher biocompatibility. Nevertheless, the PLL backbone is still widely used in newly developed polymer-based carriers due to its strong condensation ability [65].

3.2 Poly(ethylenimine)

Ever since it was first introduced for the delivery of nucleic acid drugs in 1995 [66], PEI has been recognized as one of the gold standards among nonviral carriers due to its outstanding delivery efficiency. Two different PEI polymers, branched and linear, are applicable for the delivery of nucleic acid drugs. Branched PEI is synthesized by the acid-catalyzed ring-opening polymerization of aziridine (Fig. 3) [67]. Primary, secondary, and tertiary amines exist in the branched PEI, at a ratio of 1:2:1, independent of the molecular weight [68]. On the other hand, linear PEI is synthesized by the cationic ring-opening polymerization of 2-substituted-2-oxazoline monomers and the hydrolysis of the amide groups in the resulting poly(2-substituted-2-oxazoline) (Fig. 3) [69, 70].

PEI has a structure with one amine nitrogen in every three backbone atoms, which can be protonated during the acidification. The highest amine density of PEI among cationic polymers has been supposed to be the main reason for its high delivery efficiency. Two possible mechanisms were proposed in order to rationalize the relationship between the amine density and the high delivery efficiency. The pH-dependent protonation of the branched PEI inspired the hypothesis of “endosome buffering” or the “proton sponge” mechanism (Fig. 4) [66]. Only 11% of amine residues in the branched PEI are protonated at pH 7.5, but a further

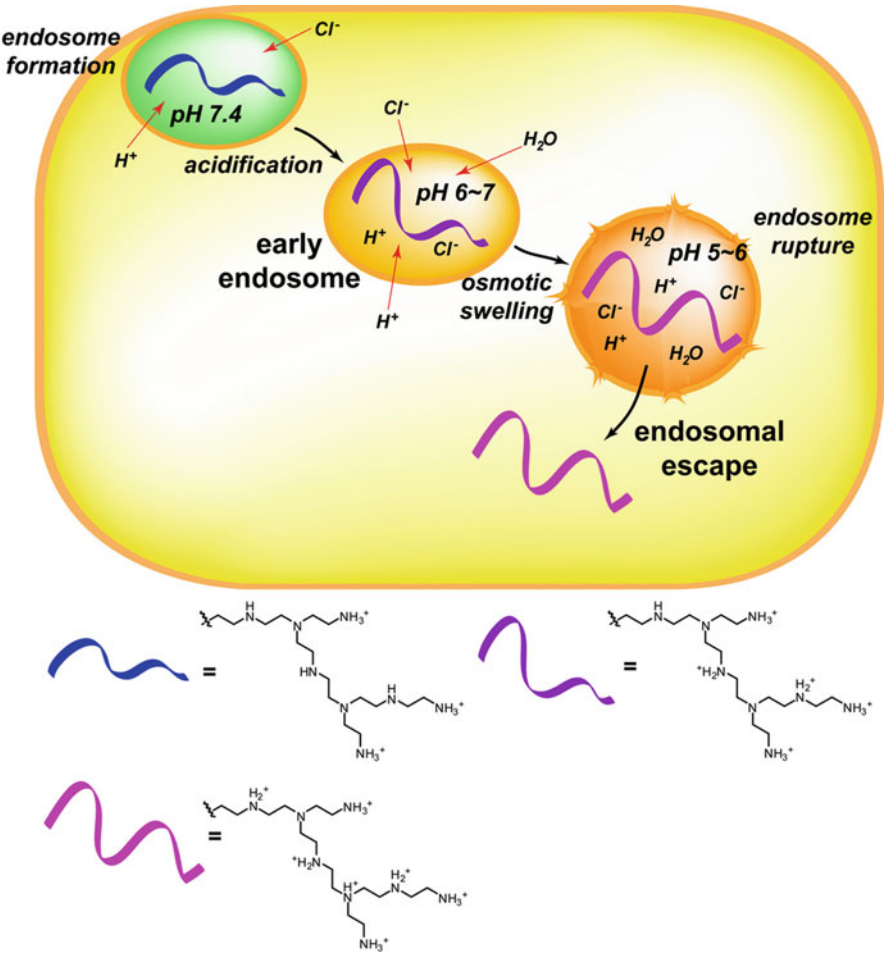

Fig. 4 Endosome escape mechanism for branched poly(ethylenimine) according to the endosome buffering hypothesis

27% can be protonated during the acidification to pH 5 [71]. As mentioned in Sect. 2.3.1, the proton pumps in the endosomal membrane cause a decrease in endosomal pH from 7.4 to 4.8. The high buffering capacity of the branched PEI could perturb the rapid acidification, so that a greater influx of protons would be required for the maturation from endosome to lysosome. Because the influx of protons is usually followed by the passive influx of chloride ions to maintain charge neutrality, an extraordinarily large ionic influx could cause osmotic swelling and subsequent disruption of the vesicular structure of the endosome. As a result, nucleic acid drugs complexed with branched PEI can escape the endosome before degradation by lysosomal nucleases.

In contrast, the linear PEI, whose amines are almost secondary, showed protonation of over 70% at pH 7.4 [72]. Therefore, the high delivery efficiency of the linear PEI cannot be rationalized by the proton sponge hypothesis alone. It was suggested that PEI could rupture the endosome by direct interaction with the endosomal membrane [40]. The destabilization of the membrane by direct contact was also supported by the result that a polymer with *N*-(2-aminoethyl)-2-aminoethyl functionalities showed both pH-dependent membrane destabilization and high DNA delivery efficiency [73].

Similar to the case of PLL, complexation with PEI can protect nucleic acid drugs from nuclease attack. The major bottleneck of PEI-based carriers for DNA delivery is probably entry into the nucleoplasm because their endosomal escape is highly efficient [74]. Only a small portion of the pDNA can reach the nucleoplasm, and the efficiency is further decreased when the cell cycle is synchronized to the early G1 phase [47]. Linear PEI has shown less dependence of delivery efficiency on the cell cycle than has branched PEI [51].

The molecular weight of PEI influences the delivery efficiency of nucleic acid drugs. High molecular weight PEI is recommended for the efficient delivery of pDNA [75]. On the other hand, low molecular weight PEI of less than 2 kDa showed higher efficiency in the delivery of shorter nucleic acid drugs such as mRNA [76]. The difference in the stability in the cytosol is probably the main reason for the difference in behavior. High molecular weight PEI can provide sufficient stability for the pDNA complexes until nuclear entry, but this stability is too high for mRNA, which must be released into the cytosol to be biologically active.

The delivery efficiency is also highly dependent upon the N:P ratio. High molecular weight PEI can form a complex with pDNA at stoichiometric N:P ratios > 1. At a higher N:P ratio, the complex can be stabilized by the electrostatic charge repulsion, and the transfection efficiency is increased. The optimal N:P ratio for the highest transfection efficiency varies according to the target cell, and scanning for the optimal N:P ratios is required in most in vitro and in vivo experiments. In most cases, N:P ratios from 4 to 20 have been selected.

Although PEI is one of the most efficient carriers for nucleic acid delivery, its high cytotoxicity significantly limits its clinical application. The cytotoxicity of PEI is likely to originate not from the complexes but from free PEI polymers [77]. Because PEI has been shown to destabilize the lipid membrane [78], free PEI can generate acute cytotoxicity within 2 h of treatment with a PEI–pDNA complex. Following this acute phase, late cytotoxicity was induced by PEI molecules detached from the complexes after the release of the pDNA. It was also suggested that PEI can alter the transcription process of other genes by interaction with the host chromosome in the nucleus. Generally speaking, high molecular weight PEI showed much higher cytotoxicity than low molecular weight PEI. Therefore, the molecular weight of 25 kDa (branched PEI) or 22 kDa (linear PEI) was selected to obtain both high delivery efficiency and tolerable cytotoxicity with PEI.

Despite the relatively high cytotoxicity, the outstanding delivery efficiency of PEI has attracted the attention of researchers searching for an ideal delivery system

for nucleic acid drugs. Various methods to reduce cytotoxicity while maintaining the endosomal escape have been attempted. Inspired by the low cytotoxicity of low molecular weight PEI, researchers have attempted crosslinking it with various biodegradable bonds [79]. Crosslinked PEI showed high DNA delivery efficiency as well as low cytotoxicity due to rapid degradation of the biodegradable bonds and the resulting decrease in molecular weight. Grafting of biocompatible polymers such as PEG is another important strategy to reduce the high cytotoxicity of PEI [80].

3.3 Poly(amidoamine) Dendrimer

A dendrimer is a type of polymer that has a well-defined branched backbone. Stepwise synthetic procedures produce dendrimers with very narrow molecular weight distributions and polydispersities (M_w/M_n) below 1.10 [81]. Because the synthetic procedure allows for the precise control of size, shape, and functional groups, dendrimers have high potential in a wide range of scientific and technological applications.

Poly(amidoamine) dendrimers were the first complete dendrimer family to be synthesized, characterized, and commercialized [82]. PAMAM dendrimers are synthesized from an ammonia or ethylenediamine core by serial Michael additions of alkyl acrylate and amidation with ethylenediamine (Fig. 5). The resulting structure of the PAMAM dendrimer consists of internal tertiary amines and external primary amines. The *N*th generation of a PAMAM dendrimer with an ammonia core has (3×2^N) primary amines on its surface and $(3 \times 2^N - 2)$ tertiary amines in the interior. Similarly, the *N*th generation of a PAMAM dendrimer with an ethylenediamine core has (2^{N+2}) primary amines and $(2^{N+2} - 2)$ tertiary amines. The globular structure of a PAMAM dendrimer with high positive charge density is similar to that of a histone protein. The diameter of a seventh-generation of PAMAM dendrimer is about 7–8 nm, the size of a native histone cluster. The positively charged PAMAM dendrimer can induce the compaction of plasmid DNA to prevent its degradation by DNase [83]. A DNA wrapping model similar to a nucleosome (natural histone–DNA complex) was proposed on the basis of an ethidium bromide intercalation experiment [84].

There is a significant difference in the basicities of the surface primary amines ($pK_a = 6.9$) and the internal tertiary amines ($pK_a = 3.9$) of a PAMAM dendrimer [85]. Only the positively charged primary amines on the surface can participate in the compaction of nucleic acid drugs at physiological pH. The remaining tertiary amines are protonated during the acidification process in the endosome, which is expected to show an endosome buffering effect. High delivery efficiency was observed in pDNA transfection experiments with a PAMAM dendrimer in various mammalian cells [86]. Oligonucleotides such as antisense DNA was also successfully delivered [87]. It was reported that the PAMAM dendrimer–nucleotide complex was maintained intact until its delivery into the nucleus [88].

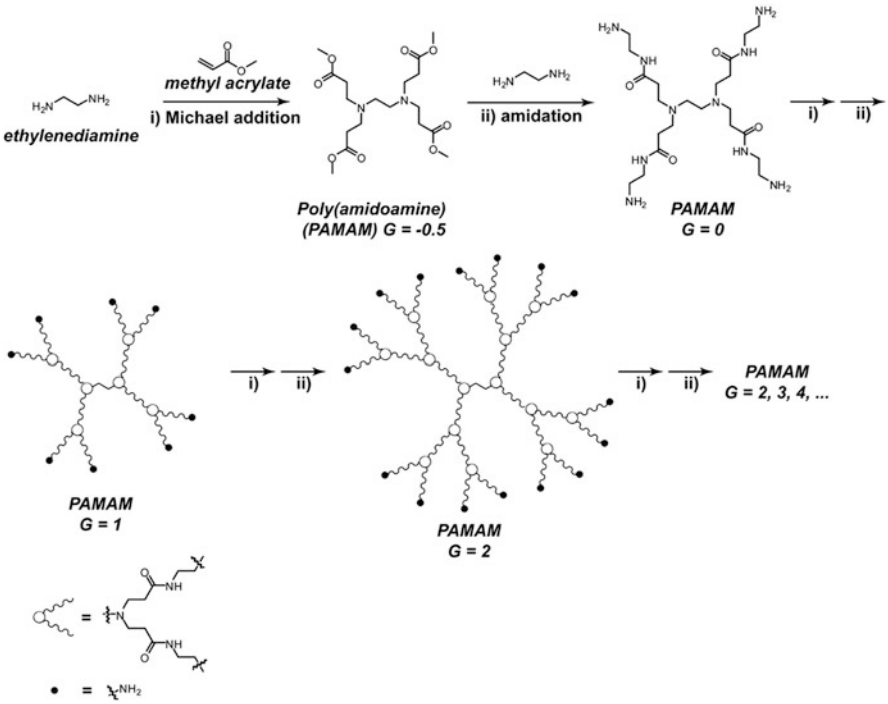

Fig. 5 Synthesis of poly(amidoamine) (PAMAM) dendrimers

The different chemical reactivities of the primary amines and the tertiary amines enable different functionalities to be introduced to the PAMAM dendrimer. For example, the carboxylate groups in lysine or arginine can be coupled only with primary amines [89]. The surface-modified PAMAM dendrimers, without disrupting the endosome buffering activity of internal tertiary amines, showed over tenfold enhanced delivery efficiency compared to an unmodified PAMAM dendrimer. Cyclodextrin was also introduced to the surface primary amines for the enhancement of delivery efficiency [90].

Poly(propylenimine) (PPI) dendrimers are another series of dendrimers that are commercially available. They are synthesized by the Michael addition of acrylonitrile followed by catalytic hydrogenation of the nitriles with Raney cobalt and hydrogen gas to primary amines [91]. They show a structural similarity to PAMAM dendrimers, with primary amines on the surface and tertiary amines in the interior, resulting in a similar DNA compaction activity and delivery efficiency into cells [92]. However, because of the relatively difficult synthetic procedure and lower delivery efficiency compared with PAMAM dendrimers, only a few reports regarding PPI-based delivery of nucleic acid drugs have been published. Surface modification of the PPI dendrimer with arginine showed a significant enhancement of delivery efficiency [93].

The size and structure of the PAMAM dendrimer family can be controlled easily and carefully. Although it was reported that heat-induced solvolysis of the perfect dendritic structure of PAMAM dendrimers was helpful for the enhancement of transfection efficiency, probably due to the more flexible structure [94], the highly controlled core–shell structure of PAMAM dendrimers can be a very attractive backbone for the detailed study of structure–function relationships in the polymeric delivery of nucleic acid drugs. The easy modification of surface functionalities should also facilitate various biomedical applications for PAMAM dendrimer derivatives.

4 Evolution of Polymeric Carriers

4.1 Overcoming Extracellular Barriers

4.1.1 Condensation and Hydrophobic Modification

Condensation with positively charged polymers is one of the easiest and most effective ways to shield nucleic acid drugs from digestion by nucleases [56]. At a charge ratio (or N:P ratio) above a certain value, positively charged polymers can condense the anionic nucleic acid drugs spontaneously. For large-sized nucleic acid drugs such as pDNA, this condensation produces PICs at sizes compatible with cellular uptake. In addition, the electrostatic interaction between positively charged polymers and small-sized nucleic acid drugs such as asDNA or siRNA can produce PICs whose molecular weights are well over the cut-off value of glomerular filtration.

However, complexes based only on electrostatic interaction are often dissociated in physiological fluids [95]. High ionic strength at the physiological salt concentration reduces the gain of free energy during PIC formation. Various charged proteins including albumin disturb the proper interaction between the charged polymers and nucleic acid drugs. Predissociation prior to arrival at the target site may be one of the most important reasons for failed delivery of nucleic acid drugs.

Therefore, many researchers have attempted to stabilize the PIC by additional stabilization forces. One such attempt has been the development of lipopolymers, which are produced by the introduction of hydrophobic moieties into existing polymers. The transfection efficiency of low molecular weight PEI was significantly enhanced by hydrophobic modification with cholesterol [96]. Conjugation with other lipids such as myristic acid or lauric acid also enhanced the transfection efficiency of PEI [97, 98]. The balance between the hydrophobic lipid groups and the charged ethylenimine moieties is crucial for the stabilization of PIC in physiological solution.

Increased physiological stability can also be observed clearly in Terplex systems [99]. A Terplex system consists of nucleic acid drugs, stearylated PLL,

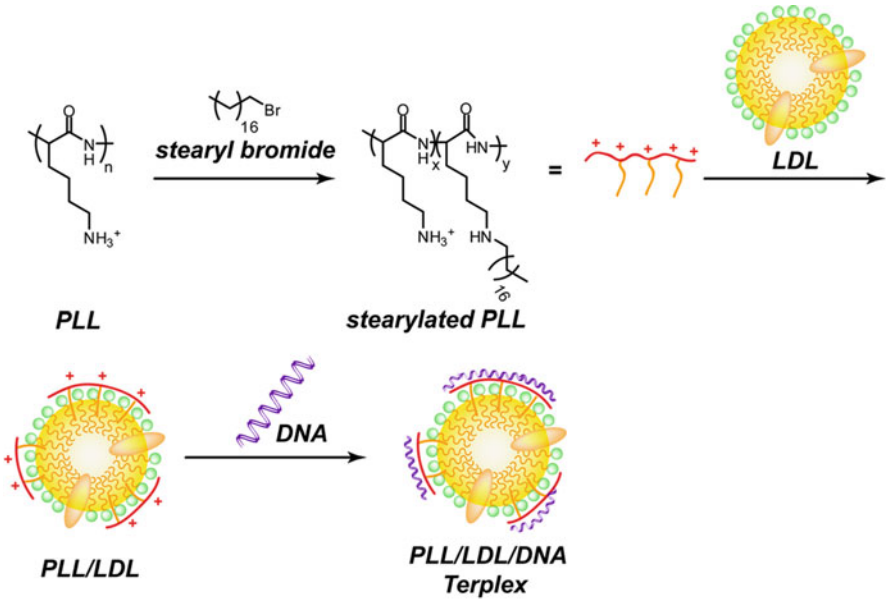

Fig. 6 Preparation of the Terplex system

and low-density lipoprotein (LDL) (Fig. 6). The primary amines in PLL were partially modified with stearyl bromide to increase the hydrophobicity of PLL. Stearyl groups can assist the intercalation of the PLL into LDL. Finally, a Terplex system was formed between the PLL-coated LDL and nucleic acid drugs. The Terplex system showed high delivery efficiency on a wide range of mammalian cells expressing LDL receptors on their plasma membrane. Due to its high stability in physiological fluids, the Terplex system achieved prolonged systemic circulation after intravenous administration [100].

The regulation of condensation via temperature cues has been investigated along with the recent development of temperature-sensitive polymers. Various polymers including poly(*N*-isopropylacrylamide) (PNIPAAM) showed a hydrophilic–hydrophobic transition at a lower critical solution temperature (LCST) [101]. The LCST of the PNIPAAM is 32°C, but the LCST is easily controllable by copolymerization with other monomers or conjugation with other polymers. When a temperature-sensitive polymer with a LCST below body temperature is conjugated with a cationic polymer, the copolymer can be expected to condense nucleic acid drugs tightly at body temperature. Indeed, PNIPAAM-grafted polyarginine showed temperature-dependent transfection efficiency [102]. PNIPAAM-grafted PEI also showed enhanced transfection efficiency at elevated temperatures [103]. Although the increase in the transfection efficiency is relatively marginal and the reason for the enhancement of the efficiency is not clear, the introduction of a temperature-sensitive hydrophobic moiety provides a new strategy for the development of carriers with controlled stability in physiological fluids.

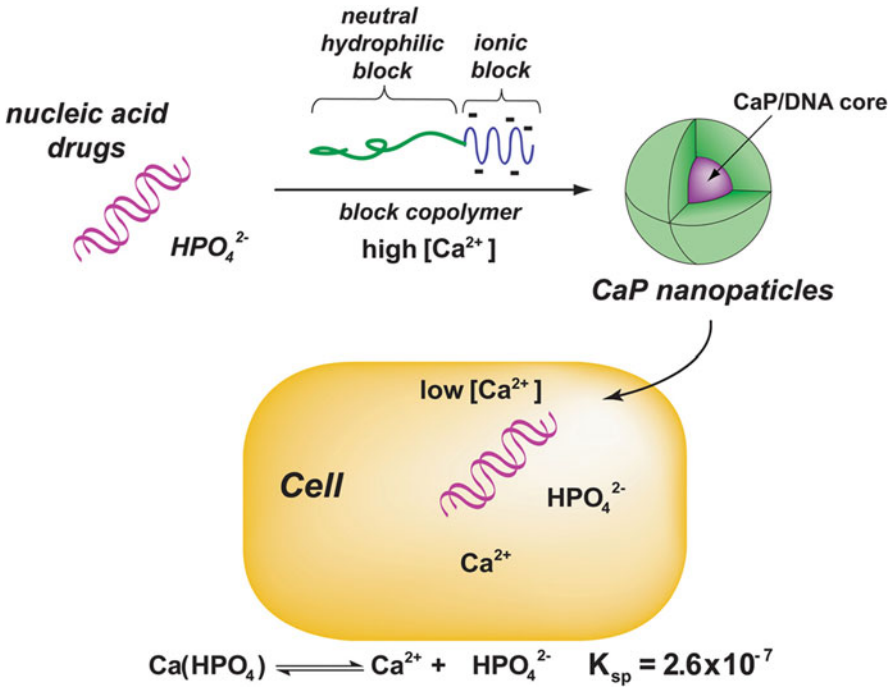

Fig. 7 Delivery of nucleic acid drugs by polymer-coated calcium phosphate (CaP) nanoparticles

Co-precipitation is quite an old technology for condensing nucleic acids. Specifically, when calcium ions and phosphate (or hydrogen phosphate) ions mix together in the presence of nucleic acids, a calcium phosphate (CaP; the chemical composition varies) precipitate is formed with co-precipitation of the nucleic acid as a substitute for the phosphate ions (Fig. 7). A DNA-containing CaP precipitate showed successful transfection to mammalian cells [9]. Although the sizes of the original CaP particles vary widely, newly developed CaP self-assembly with a palisade of block copolymers could generate CaP nanoparticles with both uniform size and stability. The CaP nanoparticles showed efficient gene expression by the delivery of pDNA [21] and gene silencing by the delivery of siRNA [104] to mammalian cells. The stabilization of inorganic assembly with organic polymers opens a new field of application in polymer chemistry for biological use.

4.1.2 Crosslinking

Crosslinking is an alternative method for preventing the premature dissociation of the complex between a nucleic acid drug and a cationic polymer. Of course, stabilization by covalent crosslinking must be reversible and carefully controlled because over-stabilization can prohibit the release of nucleic acid drugs from the

delivery system at the final target place. Therefore, biodegradable bonds have been used in crosslinking moieties for temporary stabilization and controlled release.

A disulfide bond is the one of the most frequently used crosslinkers. Glutathione, a sulfhydryl buffer in human biosystems, generates a strong reductive environment in the cytosol. Because the concentration of the reduced form of glutathione is 500–1,000 times higher than that of the oxidized form [105], most disulfide bonds are degraded rapidly through a disulfide exchange reaction with the reduced form of glutathione after its penetration into the cytosol [19]. Hence, it is expected that delivery systems crosslinked with disulfide bonds are quite stable in their extracellular oxidative condition but destabilized after uptake into cells.

Thiol residues for disulfide crosslinking can be introduced to polymer-based carriers by various methods (Fig. 8). Cysteine residues are often selected [106], and other reagents such as 2-iminothiolane or *N*-succinimidyl-3-(2-pyridyldithio) propionate (SPDP) are also commercially available for the thiolic functionalization of polymers. Disulfide bonds can be formed through oxidation in aqueous solution, but the crosslinking can be accelerated by other oxidants, DMSO, or oxygen gas [107]. The resulting disulfide crosslinking provides sufficient stability in the delivery of DNA [108] or siRNA [109], and even in lyophilization [110].

Schiff-base or imine bonds are also available as pH-sensitive crosslinkers. Because imine bonds are degradable at weakly acidic pH values, endosome-specific dissociation can be achieved by imine-based crosslinking. A simple imine-based crosslinker, glutaraldehyde, has been used to stabilize a DNA delivery system [111]. Photo-induced polymerization [112], epoxide reaction [113], and Michael addition [114] have also been reported as crosslinking methods. However, because

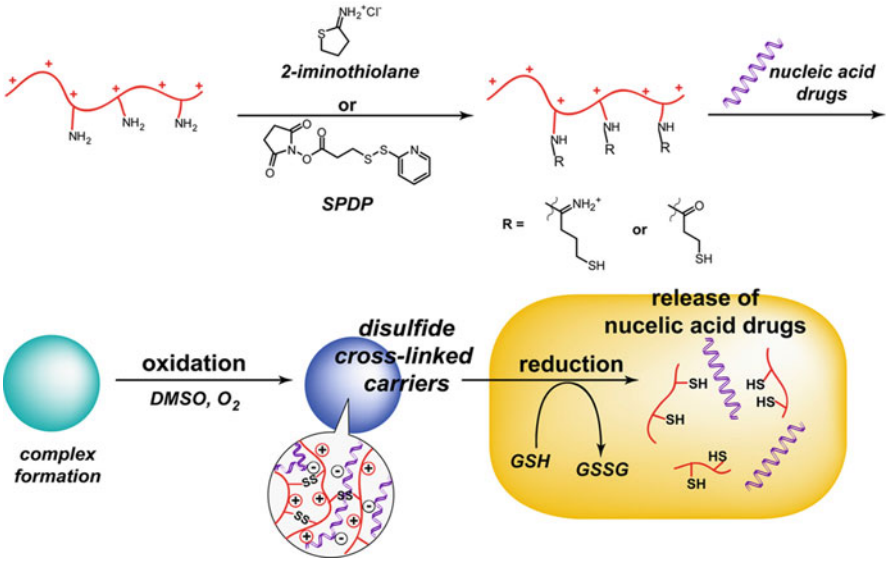

Fig. 8 Stabilization of polymeric carriers by disulfide crosslinking and the release of nucleic acid drugs in the reductive environment of the cytosol

the bonds that are newly formed via the crosslinking methods mentioned above are not biodegradable, other biodegradable functionalities such as ester [112] or disulfide bridges [113, 114] in other parts of the polymers are required for the release of nucleic acid drugs at the target site.

4.1.3 Coating

Excess amounts of positive charges are generally used for the effective condensation of nucleic acid drugs in aqueous solution. Although the excess positive charges on the surface of the PIC provide colloidal stability due to the electrostatic repulsion between the PICs, they provoke other problems in biological applications.

First, positively charged PICs show electrostatic interactions with negatively charged proteins in serum. Albumin, the most abundant protein in serum, has a negative charge at neutral pH. Severe aggregates were generated among the cationic complexes, the anionic serum proteins, and even the red blood cell surface [115]. The delivery efficiency of nucleic acid drugs was significantly reduced by aggregation, probably due to the rapid removal of the aggregates from circulation by the RES system; moreover, possible blockage of fine capillary vessels by aggregates is a limiting factor (Fig. 1) [27]. Second, the positively charged complexes sometimes induce strong cell toxicity, known as chemotoxicity [116]. Membrane destabilization by the positive charges of the PIC is believed to be one of the main reasons for the induction of chemotoxicity.

Surface coating with neutral or negatively charged polymers is one method of overcoming the weaknesses of delivery systems using cationic PIC (Fig. 9). A

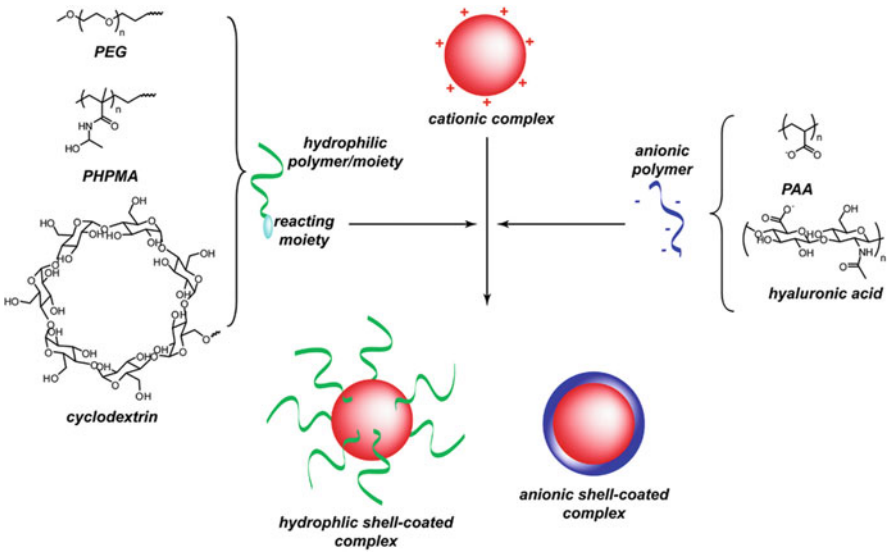

Fig. 9 Methods of coating cationic complexes with hydrophilic and anionic polymers

neutral biocompatible polymer, PEG, is frequently used as a coating polymer [115]. The large hydrodynamic volume of PEG chains can prevent direct contact between the serum proteins and the cationic polymers. Also, the chemotoxicity of a cationic polymer-based system can be reduced by PEG coating [117]. Other neutral moieties, such as poly[*N*-(2-hydroxypropyl) methacrylamide] (PHPMA) [118] and cyclodextrin [119], were also used to reduce aggregation and cytotoxicity.

Coating with negatively charged polymers is relatively simple compared to coating with neutral polymers, which requires covalent bonds between the two polymers. Because negatively charged polymers can be adsorbed on the surface of cationic PIC spontaneously by electrostatic interaction, the shielding of the cationic surface can be achieved by a simple mixing process. Poly(acrylic acid) (PAA) [33] and hyaluronic acid [120] were reported to increase the serum stability of PIC. However, PICs with a strongly negative surface are easily trapped by Kupffer cells [28], so that careful control of surface charges is a key factor in obtaining successful results. The alternative stacking of positively charged polymers and negatively charged polymers can generate layer-by-layer structures, which have been reported to improve delivery efficiency and biocompatibility [121].

4.1.4 Encapsulation

Although the most widely used method to protect the nucleic acid drugs from possible external attack is electrostatic condensation with cationic polymers, encapsulation with neutral or hydrophobic polymers can also be used. In the latter case, the nucleic acid drugs are just physically trapped in a polymer matrix, and the main stabilization force of the polymer matrix is the passive hydrophobic interaction between the polymers in aqueous solution. Because there is no strong interaction between the polymer and the nucleic acid drugs, the encapsulation of nucleic acid drugs in the polymer matrix cannot be obtained by simple mixing, but only by more complicated fabrication methods.

Polyester-based polymers such as poly(lactic-*co*-glycolic acid) (PLGA) [122] are mainly used as encapsulating polymers. The encapsulation of nucleic acid drugs is accomplished by a double emulsion method due to the difference in the water solubility of the nucleic acid drugs and the polymers (Fig. 10) [15]. In many cases, the double emulsion method requires vigorous stirring, homogenization, or a sonication process for formation of the emulsion. As the mechanical stress and the exposure to organic solvent often deactivate the nucleic acid drugs, the development of a milder encapsulation process will help to overcome the weakness of this technique.

Particles with hydrophobic surfaces can be recognized by the mononuclear phagocyte system (MPS) and cleared from the blood stream [123]. Therefore, hydrophilic polymers including PEG [124] and poly(ethylene oxide)-*b*-poly(propylene oxide)-*b*-poly(ethylene oxide) (PEO-*b*-PPO-*b*-PEO; poloxamer) [125] have often been used to modify the surface property.

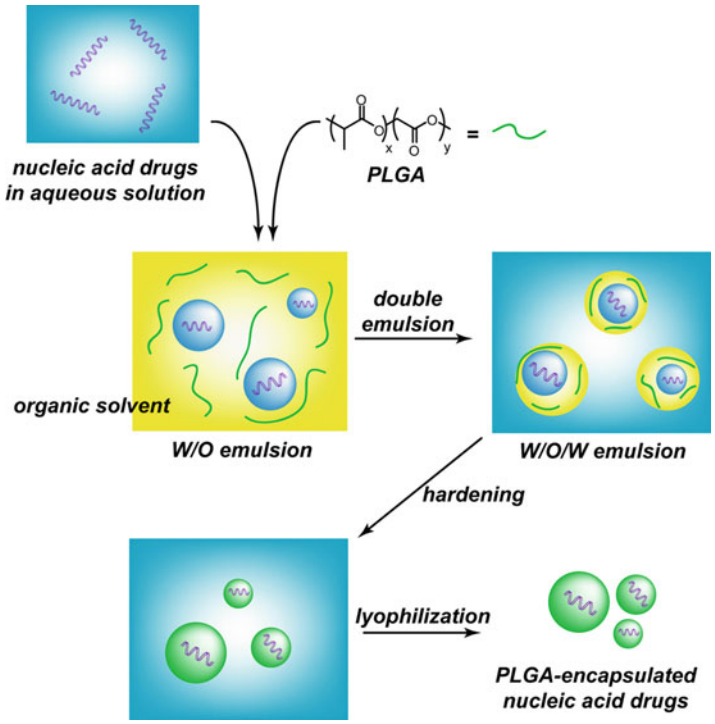

Fig. 10 Encapsulation of nucleic acid drugs in PLGA nanoparticles by a double emulsion method

The release of nucleic acid drugs from encapsulated particles is closely related to the degradation of the encapsulating polymers. As ester bonds in PLGA are slowly degraded in aqueous media, the nucleic acid drugs are released through the pores created inside the particles. The degradation or release rate can be controlled by the composition of the PLGA. The higher the hydrophobic lactic acid composition in PLGA, the slower the degradation rate [126]. However, the degradation of the ester bonds to carboxylates generates significantly lower pH in the encapsulated interior, in which condition the nucleic acid drugs can be deteriorated [127].

The development of a milder encapsulating process, biocompatible surface modification, and nonacidifying biodegradability are needed for the further improvement of the encapsulation system as a delivery vehicle of nucleic acid drugs.

4.1.5 Polyion Complex Micelles

Using a method halfway between simple electrostatic condensation and full encapsulation with neutral polymers as described above, a new delivery vehicle called a PIC micelle was developed [54, 128]. When a block copolymer with a neutral

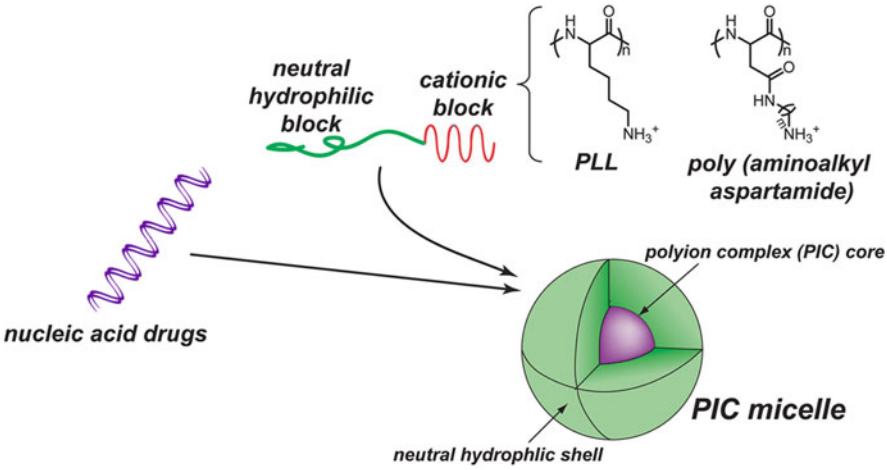

Fig. 11 Formation of PIC micelles from nucleic acid drugs and block copolymers

hydrophilic block and an ionic block is mixed with counter-charged compounds in aqueous solution, a core–shell assembly similar to a micellar structure is spontaneously formed (Fig. 11). The core of the PIC micelle is composed of the PICs between the ionic block and counter-charged compounds, and the shell consists of the neutral hydrophilic block. The main driving force for the formation of PIC micelles is the electrostatic interaction between the ionic block and counter-charged compounds. Because the shell completely surrounds the internal cargo and protects it from external attack, the PIC micelle has high potential as a molecular container and carrier of biomolecules. Various charged biomolecules including DNA [129], RNA [130], proteins [131], and even antibodies [132] have been reported as the internal cargos of PIC micelles for delivery into biosystems.

Biocompatibility and shielding ability are two important factors in the choice of polymers for the hydrophilic shell. Although PEG is the most frequently selected, acrylamide polymers [133], acrylate polymers [134], and methacrylate polymers [135] are also used as the neutral hydrophilic block. Owing to the development of heterobifunctional hydrophilic polymer derivatives [136], targeting ligands for the specific delivery of the nucleic acid drugs can be introduced on the surface of PIC micelles [137]. Meanwhile, cationic polymers are generally selected as the ionic block for complexation with anionic nucleic acid drugs. Poly(amino acid) derivatives including PLL [24] and poly(aminoalkyl aspartate) [73] are frequently used. Many biosignal-sensitive moieties to enhance delivery efficiency and control the release of nucleic acid drugs have been incorporated into the ionic block of the block copolymer [138].

The most attractive points of PIC micelles are their simple and efficient encapsulation of nucleic acid drugs and their high biocompatibility. However, their destabilization in solutions of high ionic strength is still a problem limiting their in vivo application. Active research on the stabilization of PIC micelles by

crosslinking [108], introduction of a hydrophobic moiety [139], and temporary increase in charge density [140] are now being performed.

4.2 Targeting and Cell Entry

4.2.1 Enhanced Permeability and Retention Effect

After escaping the extracellular barriers described in Sect. 2.1, carriers containing nucleic acid drugs have access to the target tissue. Many polymer-based carriers are based on the concept of specific delivery into a target tissue. Although the ligand–receptor interaction is the main strategy in targeted drug delivery, significant enhancement of targeting or cell entry can be achieved in some tissues, especially in tumors without any targeting ligands.

Passive targeting can be achieved by the "enhanced permeability and retention (EPR)" effect of tumor tissues (Fig. 12) [141]. Solid tumors show highly permeable characteristics due to the overexpression of the vascular permeability factor (VPF) and vascular endothelial growth factor (VEGF), and the secretion of various

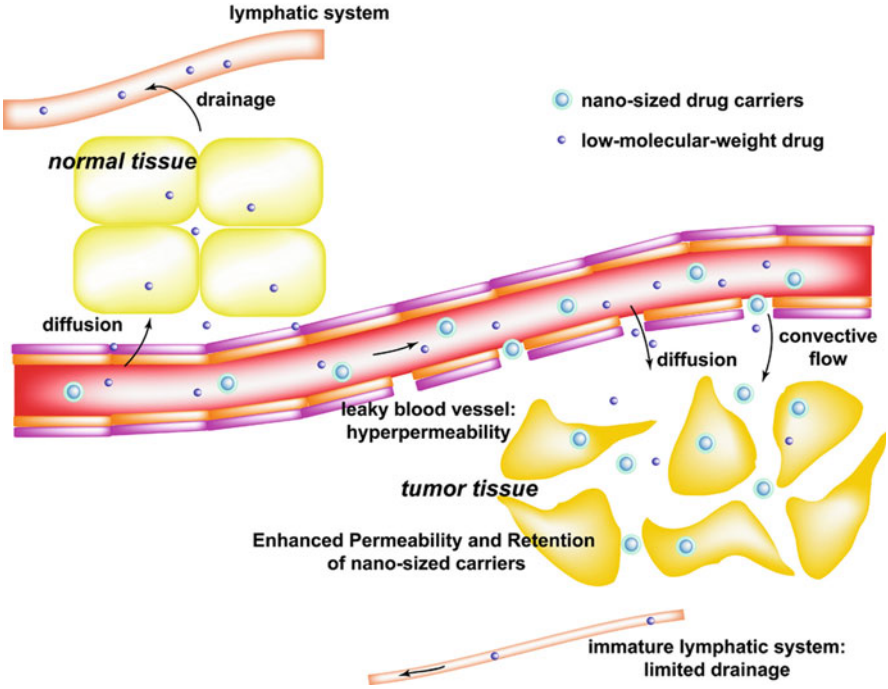

Fig. 12 EPR effect of tumor tissue. Nanosized drug carriers can accumulate in tumor tissue without any ligands

hormones including basic fibroblast growth factor (bFGF) and nitric oxide [142]. Although they have difficulty penetrating normal endothelial walls, nanosized particles can easily penetrate and accumulate in the looser tumor tissue. The vascular cut-off value of tumor tissue is around 500 nm; therefore, polymer-based carriers below this cut-off value preferentially accumulate in tumors rather than in normal tissues [143].

Furthermore, given that the RES system removes particles > 300 nm from the blood stream, the prevention of aggregates of over several hundred nanometers is essential for successful delivery into tumors. Various techniques described in Sect. 4.1 are also meaningful for passive tumor targeting.

4.2.2 Nonspecific Endocytosis

Without any ligands or helper reagents, delivery vehicles of submicrometer size can be absorbed by cells through nonspecific endocytic pathways. Generally, more positively charged carriers show more efficient internalization because the electrostatic interaction with negatively charged proteoglycans on the plasma membrane can induce nonspecific endocytosis [32]. However, as mentioned above, the positively charged surface causes problems such as aggregation in serum or chemotoxicity to the cells. By contrast, polymeric carriers with anionic or neutral surfaces exhibit limited aggregation and low toxicity, but reduced efficiency of uptake into cells. Polymeric carriers are still evolving in order to solve the contradictory problems of the surface charge effect on the safety and efficiency of nonspecific endocytosis.

One solution is the use of cell-penetrating peptides (CPPs) (Fig. 13) [144]. Originating from viral proteins [145], CPPs have 5–40 amino acid sequences with high content of basic amino acids such as arginine or lysine, and often hydrophobic amino acids. CPP incorporation or conjugation to the delivery carriers facilitates nonspecific entry into the cytosol, although the entry mechanism is quite controversial [146]. Selective ionic or hydrogen bonding between the arginine residues in CPPs and phospholipids on the plasma membrane could be an important factor of CPP-based entry [147, 148]. The induction of endocytosis, direct penetration through the plasma membrane, and the formation of pores in the plasma membrane may all be possible mechanisms. Although a possible immune response against the conjugation of the virus-derived CPP is one limitation of this method, CPPs have great potential in the delivery of biopharmaceuticals, including nucleic acid drugs.

The introduction of a lipophilic moiety into cationic polymers provides a way to reduce the cationic charge density of the polymeric carriers while maintaining high delivery efficiency. Lipophilic or hydrophobic moieties that are introduced to the polymer-based carriers can directly interact with the plasma membranes of cells to induce the uptake of the carriers into the cytoplasm [98]. The thermosensitive polymer PNIPAAM can be used as a hydrophobic moiety. PNIPAAM acts as a hydrophilic moiety below the LCST point, but turns into a hydrophobic moiety that destabilizes the plasma membrane for efficient internalization at temperatures

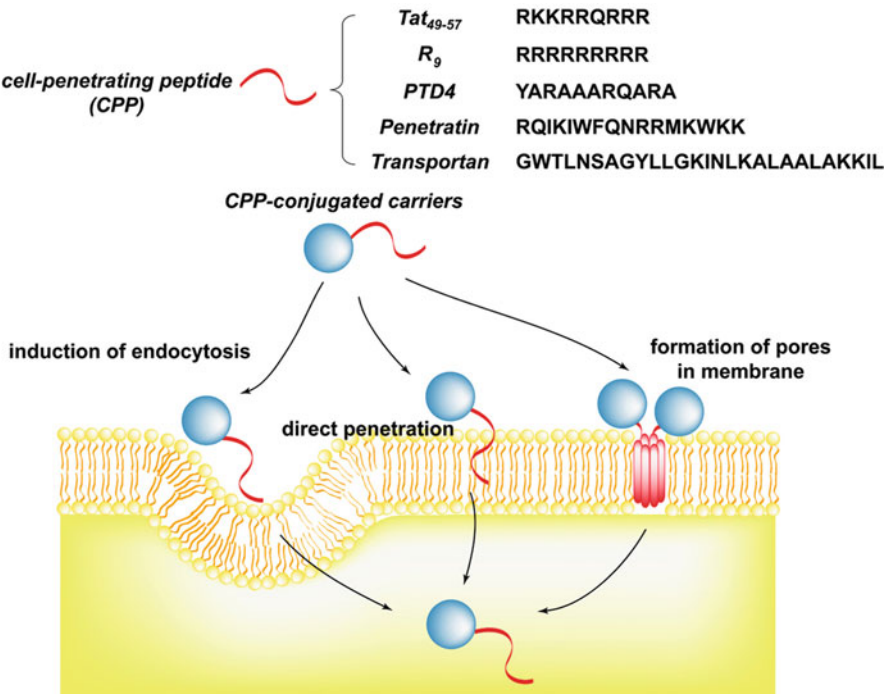

Fig. 13 Amino acid sequences of some cell-penetrating peptides (CPP) and translocation of CPP-conjugated carriers across the plasma membrane

above the LCST, which may be induced either by intrinsic heat from inflammation or active metabolism, or by external irradiation (Fig. 14) [18, 103]. The development of a more sensitive polymer that responds to small changes of temperature would enhance the applicability of this system.

Another solution is site-specific deshielding near the target sites. Although a coating with negatively charged polymers or hydrophilic neutral polymers can significantly increase the biocompatibility of the cationic polymer-based carriers, a decrease in surface positive charges may reduce the efficiency of cell entry. If the coated polymers can be deshielded at a target site, the regenerated positive charges can facilitate nonspecific entry into the cells at a specific site. It was reported that the concentration of glutathione near cancer tissues is seven times higher than that in normal tissues [149] and that plasma-membrane-associated protein disulfide isomerase (PDI), which is strongly related to disulfide cleavage, is constitutively activated in cancer cells [150]. Therefore, the coating of cationic polymer-based carriers with neutral or negatively charged polymers based on disulfide linkages has the potential to enhance internalization selectively at the cancer tissue [151]. A pH-sensitive deshielding [17] can also be an effective method to increase the cancer-specific enhancement of nonspecific entry, considering that cancer and inflammatory tissue have a lower pH than normal tissue [152].

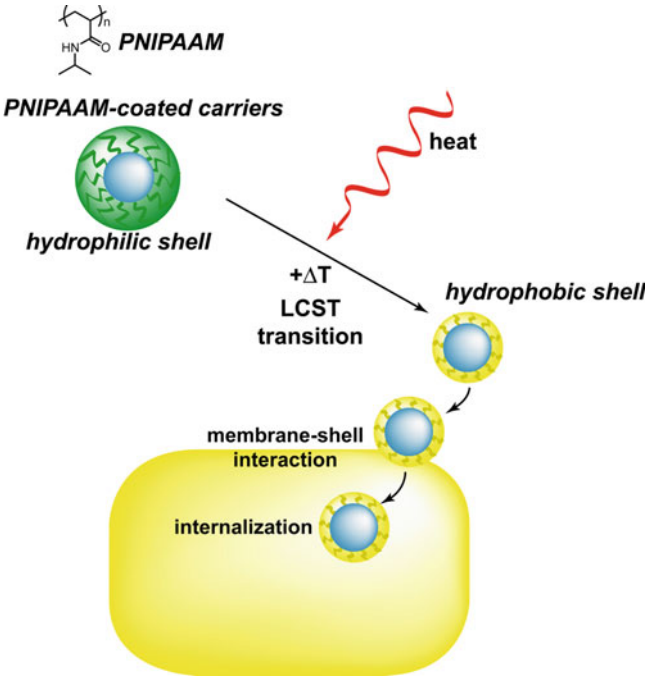

Fig. 14 LCST transition of a thermosensitive polymer, PNIPAAM, and the enhanced internalization of the PNIPAAM-coated carriers by temperature increase

4.2.3 Ligands and Receptor-Mediated Endocytosis

The main strategy of the specific delivery of nucleic acid drugs is based on the ligand–receptor interaction although the EPR effect and nonspecific endocytosis can be useful (Fig. 15). For specific delivery, a ligand that shows enhanced expression in a target cell line should be selected carefully, and introduced into the delivery carriers. One of the first ligands applied to the specific delivery of nucleic acid drugs was transferrin, an iron-transporting plasma protein. Expression of the transferrin receptor is upregulated in rapidly dividing cancer cells, although the receptor is also expressed in other cells. Cancer targeting was reported to be achieved by the introduction of the transferrin ligand on PLL or PEI [8, 153]. The expression of other receptors is also enhanced in cancer cells due to the high metabolic state. Folate and epidermal growth factor (EGF) ligands are often selected for the targeting of the corresponding ligands in cancer cells [154, 155].

Of course, variant types of cells other than cancer cells can be targeted by different ligands. Hepatocytes can be targeted by galactose or lactose ligands [156, 157], and macrophages or dendritic cells by a mannose ligand [158, 159]. Epithelial cells have been targeted by RGD (arginine-glycine-aspartic acid) peptide ligands [160, 161].

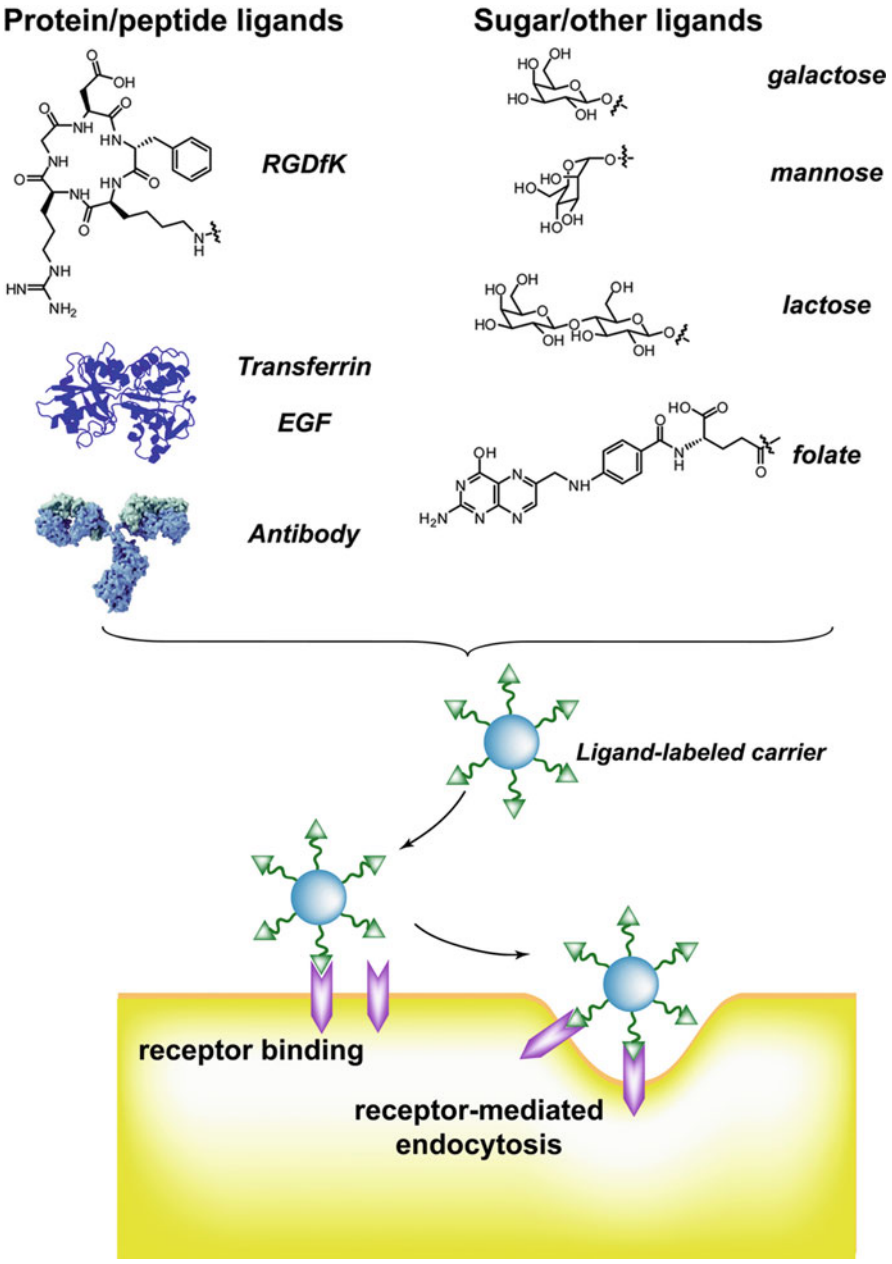

Fig. 15 Targeting ligands and receptor-mediated endocytosis

A monoclonal antibody for a certain cell surface marker can also be used as an efficient targeting ligand. Due to the specific recognition of the antigen by the monoclonal antibody, the specificity in antibody-based targeting is usually higher

than that of other ligand-based targeting. Human T-cell lymphocytes representing CD3 T cell receptors could be selectively targeted by the anti-CD3 monoclonal antibody [162]. Other leukemia cells were also targeted by an anti-JL-1 antibody [163]. CD4+ Jurkat cells [164], pancreatic islet beta cells [165], and HER2-representing tumors [166] are all targeted by the corresponding antibodies. A single-chain antibody without the Fc fragment of the antibody showed higher specificity and prolonged circulation compared to the normal antibodies, which could be recognized by macrophages and cleared from circulation [167].

The specificity of targeting is significantly reduced by cationic surface charges of the delivery carriers because nonspecific uptake is facilitated by positive charges. Therefore, the adjustment of the surface charge by coating with anionic polymers or neutral hydrophilic polymers is required for the enhancement of the specificity. Both coating and the introduction of ligands can be accomplished by a heterobifunctional neutral hydrophilic polymer linker [137]. The conjugation chemistry, the length of the spacer between the ligands and the carriers, and the ligand density on the carrier should be carefully considered so as not to interfere with the proper interactions between the polymers and nucleic acid drugs, or the ligands and the receptors [168].

4.3 Overcoming Intracellular Barriers

4.3.1 Endosomal Escape

Most polymer-based carriers for the delivery of nucleic acid drugs must escape the endosomes before complete acidification, which activates lysosomal digestion. After the discovery of the powerful endosomal destabilization activity of PEI [66], many polymer-based carriers have mimicked the structure of PEI for endosomal escape. As explained in Sect. 3.2, the proton-sponge effect of unprotonated tertiary amines and direct contact of protonated polyamines with the endosomal membrane are two possible mechanisms of endosomal disruption by PEI. Because pH-dependent protonation is critical in both mechanisms, polymers with a high density of protonable amines during the early endosomal acidification from pH 7.4 to 5.5 are one of the main kinds of polymer-based carriers with an endosomal escape function. Like tertiary amines in PEI, protonable moieties with low pK_a values have been frequently introduced into the polymer-based carriers. An imidazole moiety with pK_a of around 6.0 was one such candidate. The introduction of polyhistidine with an imidazole moiety on a PLL backbone showed significant increase in endosomal escape efficiency [169].

Because cationic amines are also required for the condensation of anionic nucleic acid drugs, some polymer-based carriers separate the functions of condensation and endosomal disruption in moieties with high pK_a and low pK_a, respectively. For example, the PLL block in the ABC-type block copolymer, PEG-*b*-poly[(3-morpholinopropyl)aspartamide]-*b*-poly(L-lysine) (PEG-PMPA-PLL), condenses

the nucleic acid drugs at neutral pH, but the remaining PMPA block buffers the protons during the acidification process [65]. Similarly, the PAMA block in PEG-*b*-poly(silamine)-*b*-poly[2-(*N*,*N*-dimethylamino)ethyl methacrylate] (PEG-PSAO-PAMA) functions as the condensing part, while the PSAO block serves as the buffering part [170].

The pH-dependent conformational change of peptides can be used for the enhancement of endosomal escape. Inspired by the membrane destabilization activity of CPPs, researchers have developed new peptides with an endosomal membrane-destabilizing activity. The peptides exist in a random coil conformation at neutral pH, but undergo a structural transition to an amphiphilic α-helical conformation, which penetrates the endosomal membrane leading to endosomal disruption. A 30-amino acid sequence with glutamic acid-alanine-leucine-alanine (GALA) repeats was designed to improve the pH-dependent destabilization of the endosomal membrane [41]. The glutamic acid side chains show a transition from a hydrophilic deprotonated state to a hydrophobic protonated state, which induces the conformational change of the whole peptide sequence. A similar peptide sequence with lysine-alanine-leucine-alanine (KALA) repeats was developed for both DNA condensation and membrane destabilization [171]. The cationic lysine side chain can function as the moiety for the DNA condensation. A pH-sensitive hydrophilic–hydrophobic transition and resulting endosomal destabilization were also achieved by nonpeptide synthetic polymers. Copolymers with alkyl acrylates and acrylic acids showed similar endosomal disruption activity [172]. The functions of the acrylic acid moiety and the alkyl acrylate moiety are probably similar to those of the glutamic acid and the hydrophobic amino acid in GALA, respectively.

As described in Sect. 4.1.3, the cationic surface of polymer-based carriers is often coated with neutral or anionic polymers to prevent aggregation in serum and improve biocompatibility. However, direct interaction between the endosomal membrane and the cationic polymers with endosomal disruption activity can be prevented by the coating, resulting in limited endosomal destabilization. A charge-conversion method was recently developed to solve the coating problem [173]. *Cis*-aconitic acid amide moieties exhibit negative charges due to the carboxylate groups at neutral pH, but at early-endosomal pH they degrade rapidly to expose amines with positive charges, which can efficiently destabilize the endosomal membrane. The charge-conversional polymeric carriers showed efficient and nontoxic delivery of DNA into primary cells without aggregation in serum. A silica-coated polyplex was also developed to obtain both stability in serum and efficient endosomal escape [174].

Facilitation of endosomal escape can be obtained without using pH sensitivity. Photosensitizers like porphyrins and related compounds generate singlet oxygen ($^1O_2$) by illumination (Fig. 16) [175]. This powerful oxidant damages various cellular structures including membranes, but the range of the damage is very limited due to the short lifetime of the singlet oxygen. Therefore, if these photosensitizers were incorporated into polymer-based carriers and internalized into endosomes, the endosomal membrane could be selectively destabilized by illumination, facilitating the escape of the polymer-based carriers into the cytosol [176]. Careful spatial

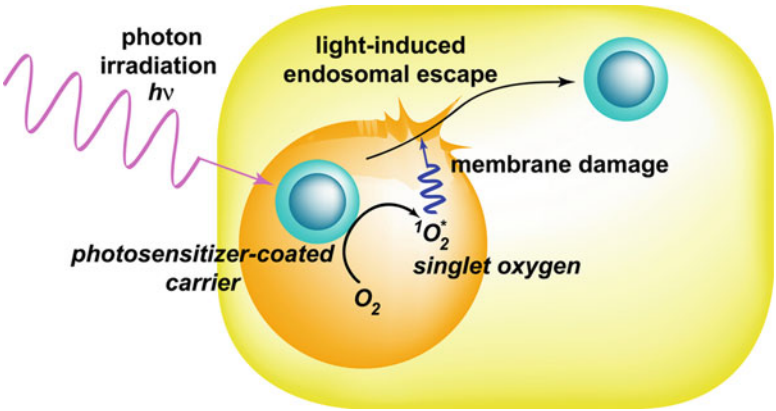

Fig. 16 Endosomal escape by photo-illumination

control is the most attractive point in such a photochemical delivery system; the effectiveness and safety of nucleic acid drug-based therapeutics will be greatly enhanced by this technology.

4.3.2 Release and Cytosolic Transport

Carriers of asDNA and siRNA should release their cargos after escaping from the endosome because the cytosol is the functional site of these nucleic acid drugs. The release of nucleic acid drugs usually occurs simultaneously with the disintegration or disassembly of the polymer-based carrier system. Biodegradable bonds can enhance the cytosolic disintegration of the polymer-based carriers. The ester bond is one of the most commonly used such biodegradable bonds (Fig. 17). An ester analog of PLL, poly[α-(4-aminobutyl)-L-glycolic acid] (PAGA), released pDNA from complexes with the degradation of ester bonds in aqueous solution, whereas PLL maintained the complexes even after 4 days [64]. Poly(β-amino ester)s, which are easily synthesized by Michael addition reactions between amines and diacrylate esters [177], were degraded by hydrolytic reaction at physiological pH to release nucleic acid drugs. A more pH-sensitive ester linkage, β-thioester, was introduced for the release of siRNA from the polymer-based carrier [178]. Phosphoester-based bonds are also used for the sustained release of nucleic acid drugs. Compared to the relatively fast degradation of ester bonds, phosphoester linkages showed a slower degradation rate ($t_{1/2} = 100$ h) [179]. Hence, a delivery system based on phosphoesters is compatible with the objective of slow release for prolonged gene expression [180].

For the selective release of nucleic acid drugs in cytosol, disulfide is probably the most suitable bond among the various biodegradable bonds. As explained in

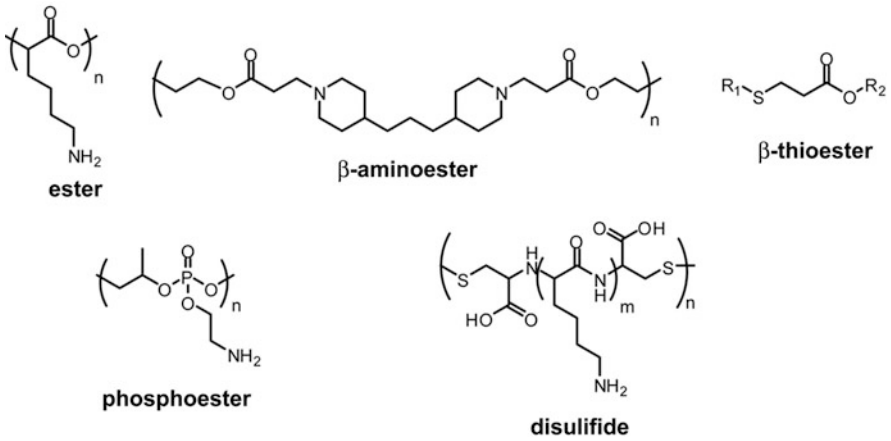

Fig. 17 Representative biodegradable bonds used in the delivery of nucleic acid drugs

Sect. 4.1.2, the reductive condition and the disulfide–thiol exchange reaction by glutathione leads the fast and selective degradation of disulfide bonds in the cytosol [19]. A PIC stabilized by disulfide crosslinking was selectively destabilized in cytosol to release the nucleic acid drug cargo [108]. The release of nucleic acid drugs was also induced by the molecular-weight-lowering of disulfide-based PLL [106] and PEI [79] in the cytosol.

The release of nucleic acid drugs is essential for the active function of nucleic acid drugs. The RNA interference (RNAi) activity of siRNA delivered by a reducible poly(amido ethylenimine) (SS-PAEI) was significantly higher than that of linear PEI [181]. The fast degradation of disulfide bonds in the SS-PAEI backbone and the resulting efficient release of siRNA can increase the bioavailability of siRNA and its accessibility to RNA-induced silencing complex (RISC), whereas linear PEI–siRNA complexes maintained their ordered structure even at 5 h after cytosolic internalization.

Meanwhile, pDNA should be delivered into the nucleus, its functional site, through cytosolic transport. Although relatively short DNA or siRNA seem to have no problem with free diffusion in cytosol, long DNAs of over 250 base pairs show a significantly reduced diffusion rate, probably due to the gel-like cytoskeleton network [42, 43]. It was reported that positively charged particles could bind to the microtubles and migrate to the nuclear envelope [45], but active targeting by a nuclear localization signal (NLS) sequence was needed for more efficient cytosolic transport to the nucleus. Also, free pDNA was aggressively digested by cytosolic nucleases within 90 min during cytosolic transport [44]. Therefore, effective condensation for protection from cytosolic nucleases and the final release of pDNA for transcription should be carefully balanced for successful gene expression of pDNA [58].

4.3.3 Nuclear Entry

Plasmid DNA must penetrate the nuclear envelope to be transcribed for gene expression. Like a plasma membrane or endosomal membrane, the nuclear envelope constitutes an impenetrable barrier to highly charged macromolecules. Passive diffusion through the NPC is possible, but only for short DNAs of below 310 base pairs [182]. Without any assistance, only 1–2% of DNA can be transported to the nucleoplasm between the narrow gateway and the cytosolic digestion [183]. The disruption of the nuclear envelope during mitotic division is the only chance for free pDNA to enter the nucleoplasm.

To increase the efficiency of nuclear entry, researchers have been mimicking the nuclear entry of viral infection and the delivery of nuclear proteins that are translated in the cytosol and function in the nucleoplasm. Many of them use certain amino acid sequences, NLSs. Most NLSs are arginine- or lysine-rich cationic sequences. NLS-containing proteins or viral capsids can be internalized into the nucleus by binding to importin-α or importin-β (Fig. 18) [184]. When NLS signals were incorporated into delivery systems for nucleic acid drugs by electrostatic complexation [185] or covalent conjugation [186], the cytosolic transport to the nuclear envelope and the following nuclear entry was greatly enhanced. The conjugation of NLS to pDNA or polymeric carriers should be carefully controlled so as not to interfere with the later interaction between the NLS and importins. For example, the nuclear entry of a 3.3-kb DNA was greatly enhanced by conjugation of single NLS, but was inhibited by the conjugation of multiple NLSs [187].

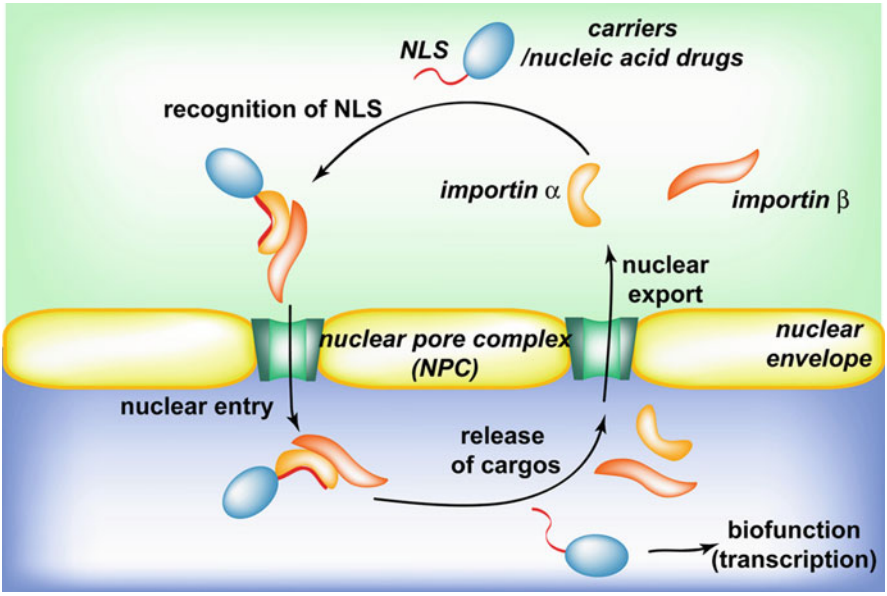

Fig. 18 Nuclear entry of carriers by NLS-mediated shuttling through the nuclear pore complex

The nucleoproteins themselves were tested for their potential as carriers of nucleic acid drugs. Because protamine [188], histone [189], and p50 [190] contain both NLS and intrinsic positive charges to condense DNA, they can be used as a substitute for synthetic polymeric carriers.

Ligands other than peptide sequences were reported to facilitate nuclear entry of nucleic acid drugs. Sugar ligands such as lactose [191] and mannose [192] showed significant improvement in nuclear transport of pDNA. The sugar-dependent nuclear translocation is supposed to involve lectins, which are sugar-binding proteins [50]. Considering that cell-surface receptors are also targets of the sugar ligands, dual targeting of a specific cell and the nucleus can be accomplished by the incorporation of a sugar ligand into a delivery system. In addition, it was reported that the nuclear entry was also enhanced by conjugation of the glucocorticoid dexamethasone [193].

The introduction of NLS and ligands is the main strategy for enhancing the nuclear entry of nucleic acid drugs, although carefully selected DNA sequences by themselves could be recognized by transcription factor proteins and internalized into the nucleus to be transcribed [194], and even a certain synthetic polymer itself showed a nuclear targeting [51, 195]. Overcoming the nuclear envelope will be a main objective of future research, especially for in vivo and clinical applications of nucleic acid drugs, because most target cells in the human body are in a postmitotic state.

5 Prospective

We have reviewed the evolution of polymer-based carriers for the delivery of nucleic acid drugs starting from the basic polymers of PLL, PEI, and PAMAM. At the beginning, research was focused on the development of efficient and safe carriers for pDNA delivery. Then, the discovery of siRNA, which can selectively control the translation of a specific mRNA, boosted the development of the siRNA delivery carriers.

An siRNA molecule has about 40 negative charges, which impede its formation of a stable complex with cationic polymers as compared with the larger pDNA. The two-turn double helix of siRNA shows a rigid cylinder-like conformation, and it cannot be strongly condensed by simple cationic polymers [196]. Inspired by the fact that the long DNA molecule can make a relatively stable complex with cationic polymers, researchers have recently attempted the elongation or pseudo-polymerization of siRNA (Fig. 19) [197]. Oligoadenines (A) and oligothymines (T) are added to the two ends of a siRNA molecule, and the sticky overhangs can elongate or pseudo-polymerize the siRNA monomers into a multimeric gene-like RNA. The complexes between the gene-like RNA and PEI have shown significantly enhanced stability, and a tenfold increase in gene silencing. By a similar strategy of elongating the siRNA using disulfide crosslinking, gene silencing was greatly enhanced in vitro and in vivo [198]. At present, a human phase I clinical trial

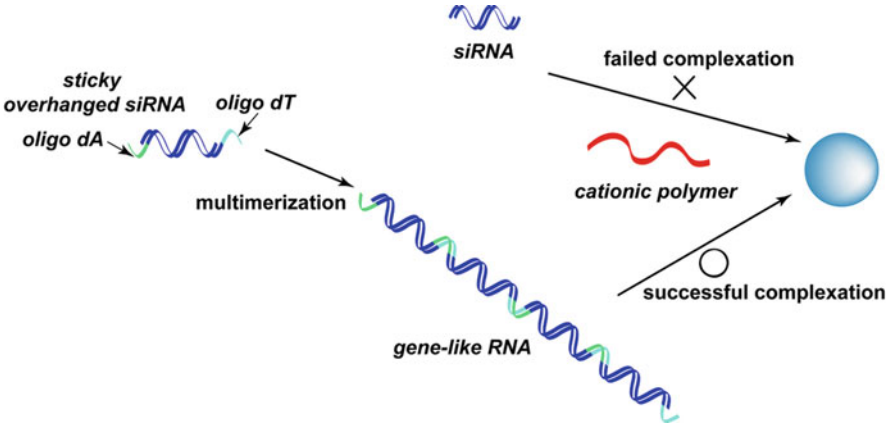

Fig. 19 Preparation of multimeric gene-like RNA from siRNAs and the formation of a stable complex

of siRNA-based therapy is being conducted via the systemic administration of polymeric nanoparticles [199].

As the practical use of nucleic acid drugs approaches realization, ensuring the safety of carriers is being emphasized. Prevention of aggregation in the blood stream, escape from excessive immune response, and reduction of cytotoxicity are all important safety objectives. The introduction of biocompatible polymers will be a possible solution for such issues as described in this review. Although the reasons why one polymer is biocompatible and another shows toxicity are still hard to answer, polymers that can be degraded into low molecular weight monomers to be metabolized in the biosystem are usually recognized as biocompatible. Various biodegradable bonds such as esters, disulfides, and amides [200], which are also important in the controlled release of nucleic acid drugs, have all been investigated for the synthesis of biocompatible polymers.

Thousands of nonviral carriers for the delivery of nucleic acid drugs have been engineered to mimic the elaborate infection machinery of viral carriers, while overcoming the weaknesses of viral transfection, and these efforts are evolving toward the development of ideal carriers. Among the various methods, polymer-based systems have become a main strategy of nonviral drug delivery due to their merits, which including the potential of adding various moieties, easy mass production, and diverse fabrication methods. Development of ideal polymer-based carriers with higher efficiency, safety, and specificity has been stimulated by recent improvements in polymer synthetic chemistry, the rapid development of supramolecular chemistry, and the accumulation of biological and clinical data on nucleic acid drug-based therapeutics.

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Aptamer: Biology to Applications

Yoshikazu Nakamura

Abstract Aptamers are short single-stranded nucleic acid sequences that are selected in vitro from large oligonucleotide libraries on the basis of their high affinity to a target molecule. Hence, aptamers can be thought of as a nucleic acid analog to antibodies. However, the potential of aptamers arises from interesting characteristics that are distinct from, or in some cases superior to, those of antibodies from several viewpoints. This review summarizes the recent achievements in aptamer programs developed in our laboratory against basic as well as therapeutic protein targets. Through these studies, we became aware of RNA's remarkable conformational plasticity, which the literature has not shed light on even though this is evidently a crucial feature for the strong specificity and affinity of RNA aptamers.

Keywords Aptamer biosensor · Aptamer therapeutics · Autoimmune diseases · RNA aptamer · RNA plasticity

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1 Introduction

The concept of using single-stranded nucleic acids (aptamers) as affinity molecules for protein or compound binding was initially described in 1990 [1–3]. The concept is based on the ability of short oligonucleotides to fold, in the presence of a target, into unique three-dimensional (3D) structures that bind the target with high affinity and specificity. Aptamers are generated by a process that combines combinatorial chemistry with in vitro evolution, known as systematic evolution of ligands by exponential enrichment (SELEX), from a complex library of randomized sequences of typically 10^{14} different molecules [4–8]. Importantly, targets of aptamers can be small (e.g., chemical compounds) or large (e.g., proteins); and simple (e.g., purified proteins) or complex (e.g., protein complexes or cell surface receptors). Therefore, aptamers can be used as reagents for affinity purification [9–11], or as biosensor elements (reviewed in [12, 13]). Moreover, the first aptamer-based therapeutic, Pegaptanib (Macugen), targeting vascular endothelial growth factor, was approved by the US Food and Drug Administration (FDA) in December 2004 for the treatment of age-related macular degeneration (AMD) [14, 15].

Considering the basic principles of aptamer selection, the high potential of RNA to create a vast set of tertiary structures is conceivable from both the "RNA world" hypothesis [16] and the concept of "molecular mimicry" between RNA and protein [17]. We have created and characterized RNA aptamers against a variety of human proteins, including human translation initiation factors, immunoglobulin G (IgG), and human cytokines, as well as the cyanine dye Cy3, and these studies have contributed to our basic understanding of aptamer potential and global applications.

2 Mechanism of Target Recognition

2.1 Anti-eIF4A Aptamer: Whole Body Capturing

Initiation of protein synthesis in eukaryotes is a highly regulated process involving at least 12 protein factors [18, 19]. The initial association of mRNA with the small (40S) ribosomal subunit requires the participation of the eukaryotic initiation factor complex, eIF4F, and the hydrolysis of ATP [18, 20, 21]. eIF4F is comprised of three subunits (eIF4E, eIF4A, and eIF4G), and binds to the cap structure (m^7GpppN, where N is any nucleotide), which is present at the 5′ end of all cellular mRNAs, via

the cap-binding protein subunit, eIF4E. The eIF4A subunit is an RNA-dependent ATPase that cycles through the eIF4F complex [22, 23]. eIF4A, in cooperation with initiation factor 4B (eIF4B), exhibits duplex RNA helicase activity [24]. Hence, the apparent role of eIF4A is to facilitate the "melting" of secondary structure present in the 5′ untranslated region (UTR) of mRNAs that would otherwise impede translation initiation [25].

eIF4A is a member of the DEAD-box RNA helicase protein family. DEAD box proteins contain nine highly conserved amino acid sequence motifs and have been implicated in a variety of biological processes involving RNA unwinding and/or rearrangement [26–29]. The crystal structure of eIF4A, either the monomer [30–32] or the dimer bound to eIF4G [33] or PDCD4 [34], exhibits a distended "dumbbell" structure consisting of two domains [31, 32, 35]. The domains probably undergo a conformational change upon binding ATP, to form a compact, functional structure via the juxtaposition of the two domains. The ATPase activity of eIF4A is stimulated by single-stranded RNA (ssRNA); however, the binding of eIF4A to ssRNA is weak, with dissociation constant (K_d) of ~100 micro M [36–38]. It is also clear that there is no defined RNA-binding module in eIF4A, in contrast to most other RNA-binding proteins [39]. This might be a general feature of DEAD-box family proteins involved in unwinding double-stranded nucleic acids [40]. Indeed, a priori, any sequence specificity would preclude the free movement of the helicase along the strand. Collectively, the molecular basis of these conformational changes is not fully understood. It was of interest to test whether or not high-affinity RNA ligands inhibited the helicase activity of eIF4A.

We used SELEX to generate an RNA aptamer, called 4Aapt, with high affinity for human eIF4A [4]. 4Aapt exhibits a dissociation constant of 27 nM for eIF4A, and sharply inhibits its ATP hydrolysis activity (Fig. 1a). Moreover, in a bicistronic construct, 4Aapt inhibits cap-dependent chloramphenicol acetyltransferase (CAT) translation, but not cap-independent, hepatitis C virus (HCV) internal ribosome entry site (IRES)-directed luciferase (LUC) translation in vitro (Fig. 1b). The binding affinity was increased by an arginine substitution in the conserved motif of eIF4A, which probably improves a predicted arginine network that binds RNA substrates. 4Aapt, however, binds much less efficiently to either of the eIF4A domains alone, when split at the linker site [4], while the two split-domains together bind significantly to 4Aapt. These findings suggest that the 4Aapt RNA interacts cooperatively with both domains of eIF4A, either in the dumbbell or the compact form, and entrap it into a dead-end conformation, probably by blocking the conformational plasticity of eIF4A. 4Aapt does not interfere with the interaction between eIF4A and eIF4G. It is assumed that the interdomain movement between the N- and C-terminal domains of eIF4A is necessary for, or coupled with, ATP hydrolysis and the helicase action [32, 41]. Therefore, we suggest that the 4Aapt RNA "staples" together the two domains of eIF4A, leading to the inhibition of its interdomain movement.

The affinity sequence of 4Aapt can be trimmed to 58 bases, the overall structure and sequence of which (nearly) seem to be required for high-affinity binding. The secondary structure of 4Aapt was largely confirmed by structural probing with

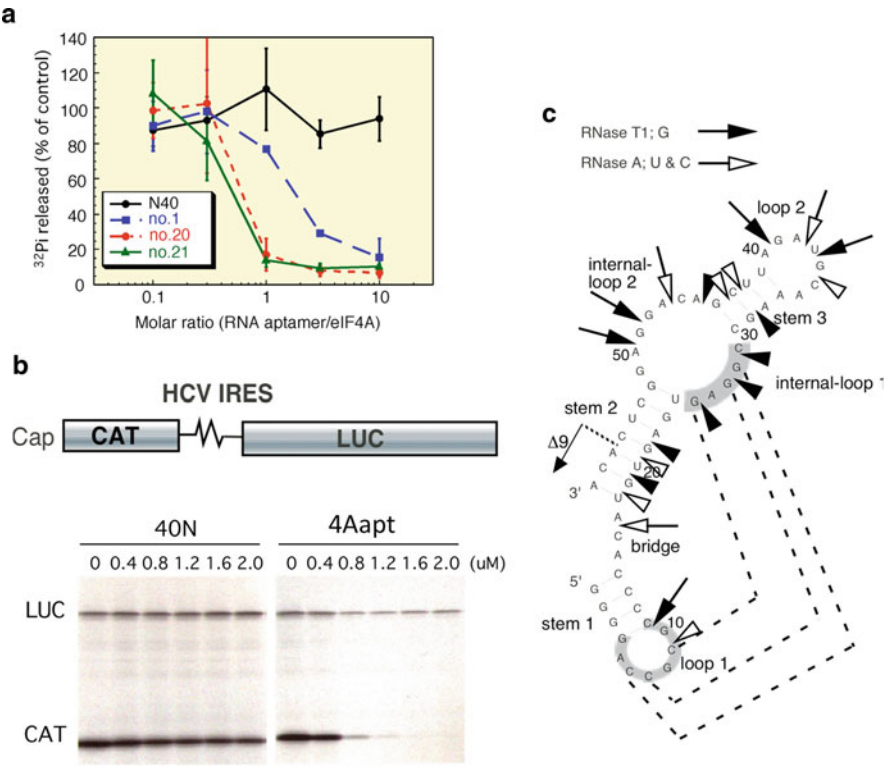

Fig. 1 Functional and structural properties of the eIF4A aptamer [4]. (**a**) Inhibition of eIF4A ATPase by 4Aapt. Inorganic phosphate (Pi) release was measured with increasing amounts of selected RNAs in the presence of saturating amounts of poly(A). Shown is the percentage of control Pi release in the absence of RNA. Four different RNAs were examined including a random 40 N pool (N40) and 4Aapt (no.21). (**b**) Inhibition of cap-dependent translation by 4Aapt. The *upper panel* shows a schematic diagram of capped CAT/HCV/LUC mRNA. The *lower panel* shows translation products of capped CAT/HCV/LUC mRNA in rabbit reticulocyte lysate. Reaction mixtures were preincubated at 30 °C for 3 min with increasing amounts (0, 0.4, 0.8, 1.2, 1.6, and 2.0 mM) of N40 or 4Aapt RNAs, followed by further incubation for 60 min at 30 °C with the addition of mRNA and [^{35}S]methionine. Products were analyzed by SDS-PAGE (15%) and autoradiography. (**c**) Secondary structure of 4Aapt RNA examined by ribonuclease sensitivity assays. *Closed* and *open arrows* indicate major cleavage points of RNase T1 and RNase A, respectively. *Arrowheads* indicate minor cleavage points. *Broken lines* between loop 1 and internal-loop 1 indicate putative base-parings

RNase T1 and RNase A (Fig. 1c). Since splitting either 4Aapt or eIF4A into two subdomains diminishes or reduces affinity for each other, it is suggested that multiple interactions, or a global interaction between the two molecules, accounts for the high affinity. To understand the structural basis of 4Aapt's global recognition of eIF4A, the solution structure of two essential nucleotide loops (AUCGCA and ACAUAGA) within the aptamer were determined using nuclear magnetic resonance (NMR) spectroscopy (see Fig. 2a) [42]. The AUCGCA loop is stabilized

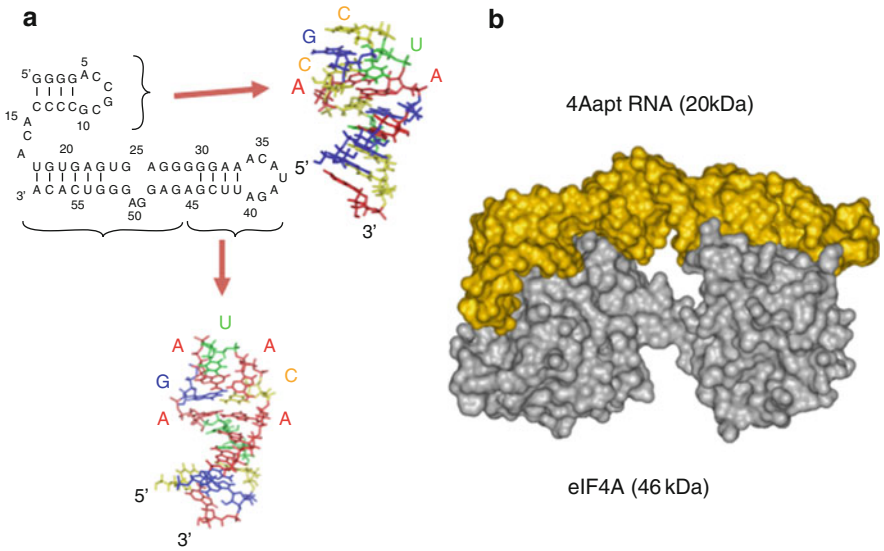

Fig. 2 Predicted structure of 4Aapt RNA. (**a**) Tertiary structures of the two subdomains of 4Aapt RNA solved by NMR [42]. (**b**) Structural model of the 4Aapt·eIF4A complex

by a U-turn motif and contains a noncanonical A:A base pair (a single hydrogen bond mismatch: Hoogsteen/Sugar-edge). On the other hand, the ACAUAGA loop is stabilized by an AUA trinucleotide loop motif and contains the other type of A:A base pair (single hydrogen bond mismatch: Watson–Crick/Watson–Crick). Taking these and other results into consideration, the structural model of the 4Aapt·eIF4A complex is presented, in which the 4Aapt RNA forms a unique structure that recognizes the global conformation of eIF4A (so-called "whole body capture"; see Fig. 2b). We assume that this wide-range interaction may be the basis for strong and specific recognition of eIF4A by 4Aapt. Considering these features, it was possible to apply 4Aapt for semiquantitative measurement of eIF4A, at nanogram levels, within whole cell lysates using a surface plasmon resonance (SPR) assay with a 4Aapt-immobilized sensor chip [43].

2.2 Anti-IgG Aptamer: Pinpoint Smart Capturing

Although the 3D structures of RNA aptamers are commonly solved by X-ray crystallography or NMR spectroscopy [44], there are only three reported high resolution structures of RNA aptamers in complex with their targets. These include RNA aptamers in complex with nuclear factor (NF)-κB solved at 2.45 Å [45], with bacteriophage MS2 capsid at 2.8 Å [46] and with thrombin at 1.8 Å resolutions [47]. NF-κB and bacteriophage MS2 capsid naturally bind to nucleic acids. The crystal structures of RNA aptamers in complex with the nucleic acid-binding

domain of these two proteins reflect these properties by mimicking naturally occurring electrostatic interactions [46, 48]. The crystal structure of an RNA aptamer in a complex with thrombin, which is not a nucleic acid binding protein, indicates that the aptamer binds to the positively charged surface of the protein that is naturally required for high-affinity heparin binding [47]. Thus, the crystal structures determined to date have suggested that RNA aptamers bind target proteins predominantly through electrostatic forces.

In contrast, we have used SELEX to obtain a 23-nucleotide RNA aptamer against the Fc portion of human IgG1 (hFc1) [49], which uniquely lacks an extensive positively charged molecular surface [50]. Therefore, it is likely that the RNA aptamer may interact with hFc1 via nonelectrostatic forces. The aptamer also exhibited remarkable species specificity to hIgG and required divalent cations for binding, as revealed by EDTA elution [49]. To investigate these remarkable properties, we solved the crystal structure of the aptamer-hFc1 complex at the 2.15 Å resolution (Fig. 3a) [51].

Several interesting features were revealed by the solved structure. First, the structure of the aptamer-bound hFc1 is superimposable upon the uncomplexed form of the structure of hFc1 (Fig. 3b), indicating that the aptamer binding caused no significant structural changes to the backbone of hFc1, and instead underlies the conformational plasticity of RNA to form a conformation that fits to the target structure.

Second, the RNA structure in the aptamer–hFc1 complex (Fig. 3b) diverges greatly from the secondary structure predicted by M-fold (Fig. 3c). Instead, the structure of the aptamer in complex forms a distorted hairpin structure with base-flipping between U6 and G7, producing a GAAA tetraloop, an internal loop, and a terminal A-form helix (Fig. 3c). The internal loop formed by this distorted structure is crucial for binding hFc1.

Third, the distorted structure is naturally unstable, and requires the presence of a hydrated calcium ion (Fig. 3d), found in the RNA major groove, that does not coordinate with protein ligands but rather binds to nonbridging oxygen atoms of the G7 phosphates in the RNA. Therefore, Ca^{2+} may also help to maintain the distinct conformation of G7, which would be crucial for binding to hFc1. These structural features were confirmed by the addition of EDTA to chelate Ca^{2+}, resulting in loss of affinity by distorting the aptamer structure. Importantly, affinity is restored upon addition of Ca^{2+} in the presence of hFc1 [51]. The reversible binding suggests reversible folding of the aptamer, achieved by the presence of the divalent cation and target hFc1.

Protein A affinity chromatography is currently the most often used procedure for purification of humanized or chimeric antibodies [52, 53], but also requires an acidic elution step that can sometimes cause unexpected aggregation or inactivation of antibodies [53–55]. Aptamer-bound IgGs are instead easily released from the aptamer resin under neutral pH conditions using simple elution buffers, such as an EDTA solution [49]. Combined with the aptamer's high specificity to hFc1, these additional potential purification advantages provides us with an alternative reagent for the mass purification of therapeutic antibodies, as previously described [49].

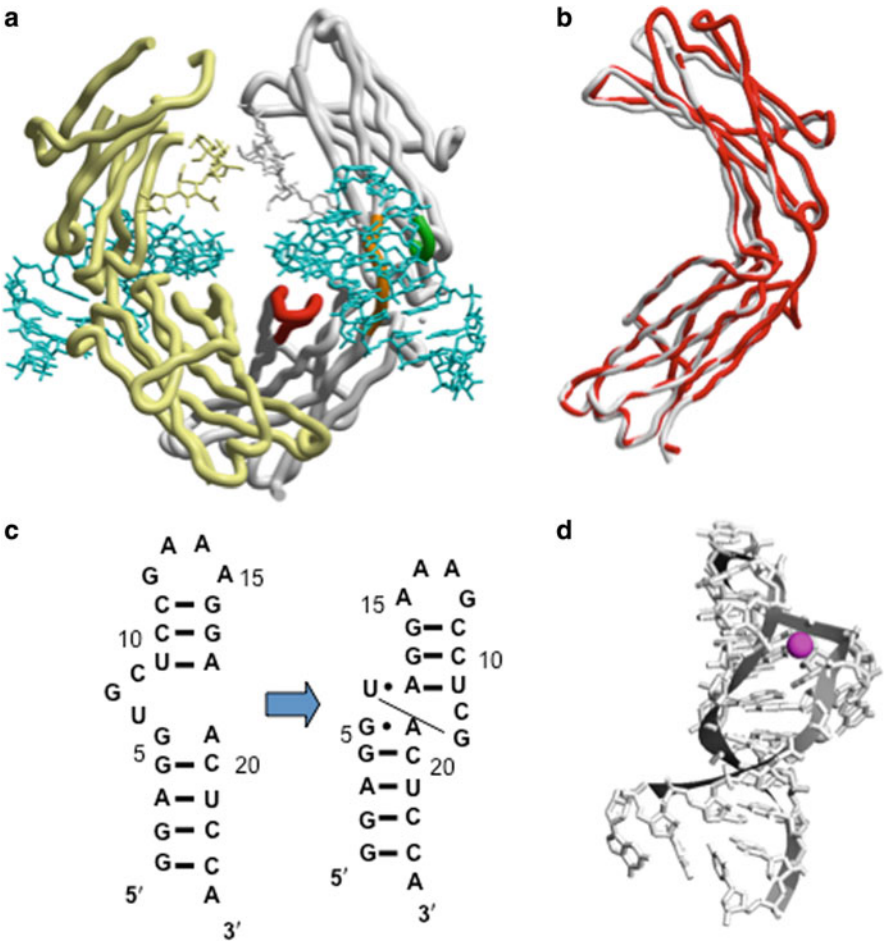

Fig. 3 Structure of anti-hFc1 aptamer and the aptamer-hFc1 complex [51]. (**a**) The 2.15 Å crystal structure of a human IgG–aptamer complex. hFc1 backbone molecules are shown in *yellow* and *gray*, and bound aptamers are shown in *blue*. Of three regions colored in *red*, *orange*, and *green* in hFc1 (*gray*), a previous study [49] suggested that the aptamer binds the *orange region* and this prediction is confirmed by the crystal structure. (**b**) hFc1 conformations uncomplexed (*gray*) and in complex (*red*) with the aptamer. (**c**) M-fold predicted secondary structure of anti-hFc1 aptamer (*left*) and its crystal structure in the complex (*right*). The global fold of the aptamer adapts a distorted hairpin structure with base flipping between U6 and G7. (**d**) Coordination sphere of Ca^{2+} (*pink sphere*). Ca^{2+} is bound in a distorted octahedral coordination environment with the phosphate backbone and five water molecules [51]

Fourth and most importantly, unlike known RNA–protein interactions, which are generally stabilized by electrostatic forces, as described above, the aptamer–hFc1 interaction is stabilized by multiple weak interactions like hydrogen bonds and van der Waals forces. For example, the stacking interaction between the aptamer's G7 and the tyrosine 373 (Y373) is crucial for the complex (Fig. 4a) and therefore the

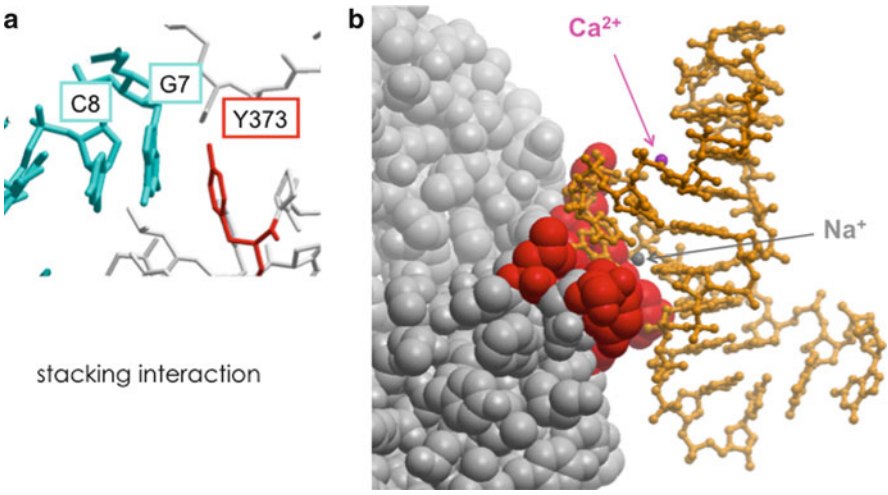

Fig. 4 Interactions between an aptamer and its target protein. (**a**) The continuous stacking interaction between hFc1 and RNA aptamer. (**b**) Enlarged view of the contact regions between hFc1 and the aptamer. The peptide region of direct interaction with the aptamer (*yellow*) is shown in *red spheres*

aptamer probably interacts through weaker forces supported by van der Waals contacts and hydrogen bonds [51]. The interaction between hFc1 and the aptamer covers 580 $Å^2$ per Fc fragment (Fig. 4b), a surface area that is relatively small compared with that of other RNA aptamer interactions (ca. 1,000 $Å^2$), but even so achieves remarkably strong affinity [51]. Therefore, it is likely that SELEX technology can select not only for molecules that interact through predominantly electrostatic forces [44–46], but also for high-specificity molecules that interact through weaker forces such as van der Waals contacts and hydrogen bonds. Together, these findings highlight the excellent conformational plasticity of RNA molecules, suggesting that RNA aptamers may be applicable to a wider range of targets than previously thought.

3 Aptamer Applications

3.1 Anti-eIF4E Aptamer for Synthetic Biology

Accurate control of translation initiation is important for cell growth. A wide range of observations have accumulated that demonstrate a causal relationship between aberrant expression of initiation factors and malignant transformation. Overexpression of eIF4E is known to cause deregulated cell growth [56, 57] and malignant transformation [58] of rodent and human cells. In fact, eIF4E levels are

a prognostic indicator of clinical outcomes in a variety of human cancers, including breast cancer as well as head and neck squamous cell carcinoma [59–61]. Hence it is conceivable that the growth promoting and transforming properties of eIF4E are due to increased translation of certain, if not all, mRNAs that are important for growth control (reviewed in [62, 63]). In fact, eIF4E overexpression does not uniformly stimulate translation of all mRNAs, but rather a subset of transcripts that are more sensitive to eIF4E levels, such as those for c-Myc [64], cyclin D1 [65–68], and ornithine decarboxylase [68–70].

Of all eIFs, eIF4E is the least abundant factor [71] and the recognition of the mRNA cap by eIF4E is the rate-limiting step of eukaryotic translation initiation. We have developed a high-affinity RNA aptamer against human eIF4E, 4Eapt1, using SELEX [72]. 4Eapt1 inhibits cap binding to eIF4E more efficiently than the cap analog m7GpppN. Consistently, 4Eapt1 specifically inhibits cap-dependent in vitro translation whereas it does not inhibit cap-independent, HCV IRES-directed, translation initiation. The interaction between eIF4E and 4E-BP1, however, was not inhibited by 4Eapt1, hence the formation of a 4Eapt1·eIF4E·4E-BP1 heterotrimeric complex that was detected by the SPR assay. 4Eapt1 is 86 nucleotides (nts) long, and the high affinity to eIF4E is abrogated by short deletions at either termini. Moreover, relatively large areas in the 4Eapt1 structure are protected by eIF4E, as determined by a ribonuclease footprinting assay (Fig. 5a). These findings indicate that 4Eapt RNAs can achieve high affinity to a specific target protein via global conformation recognition.

Interestingly, 4Eapt1, selected to human eIF4E, does not recognize yeast eIF4E (unpublished results) even though human eIF4E is able to substitute for yeast eIF4E for cell growth [73]. In accordance with the latter notion, the viable heterologous yeast strain in which the expression of chromosomal yeast eIF4E was substituted with exogenous expression of human eIF4E (see Fig. 5b) [74] became susceptible to transformation with a 4Eapt1-expression plasmid under the control of the inducible *GAL* promoter (Fig. 5b). These results provide a demonstration that aptamers can be applied to "synthetic biology" as new regulatory elements. An aptamer-based on/off switch, as shown in Fig. 5b, might prove useful for the functional study of human translation factor(s) in heterologous yeast systems.

3.2 Anti-Cy3 Aptamer as a Biosensor

Aptamers have also been generated to dyes and fluorophores, such as malachite green [75] and sulforhodamine B [76, 77]. These aptamers are composed of unmodified nucleotides and are thus applicable to sensitive, real-time detection of nucleic acid or small molecules by annealing to complementary sequences or binding to secondary aptamers to target molecules [78–80]. To reduce background fluorescence and increase detection specificity, a binary aptamer probe has been designed by Kolpashchikov based on the malachite green aptamer (MGA) [78]. MGA is unique as it dramatically increases the fluorescence of the dye [81].

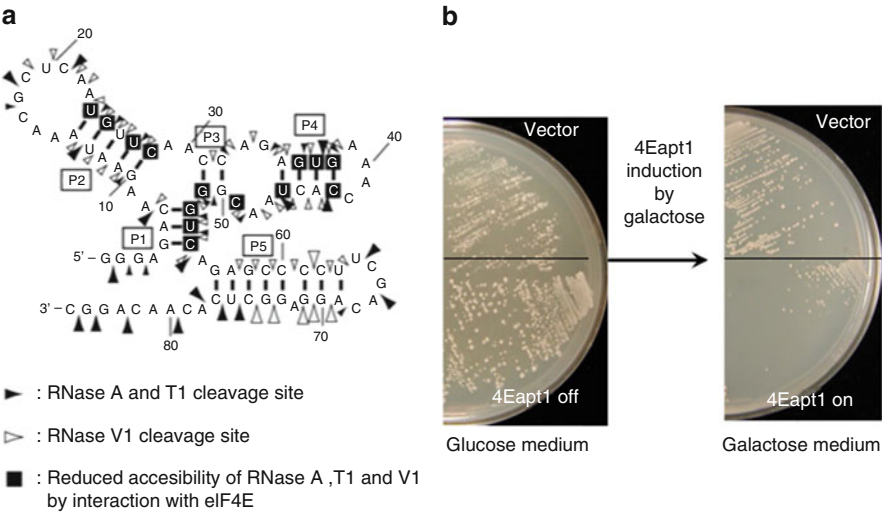

Fig. 5 An anti-eIF4E aptamer switch manipulated in yeast. (**a**) The secondary structure of 4Eapt1 examined by ribonuclease sensitivity and eIF4E protection assays. *Solid arrowheads* indicate the cleavage points with RNase A and T1, and *open arrowheads* indicate RNase V1 cleavage positions. The *arrowhead* size represents the degree of cleavage. The bases enclosed in *black squares* indicate sites protected by the addition of eIF4E from hydrolysis by RNases A, T1, and V1. (**b**) Restoration of cell growth in the *Saccharomyces cerevisiae* eIF4E-silenced strain by heterologous human eIF4E expression, and repression of cell growth by 4Eapt RNA expression [74]. The *S. cerevisiae* eIF4E gene was silenced with a *tet* promoter fusion, and human eIF4E was expressed from a plasmid. This strain was additionally transformed with a 4Eapt-expression plasmid controlled by the *GAL* promoter. Glucose medium represses 4Eapt expression whereas the addition of galactose induces 4Eapt expression, thereby reducing the viability of the strain

MGA has a stem–loop structure containing internal bulged loops [75]. These double-stranded sequences were separated into two single-strand sequences, each of which had no affinity to malachite green, and were tagged to sequences complementary to nucleic acid analytes. This probe is referred to as a binary MGA probe, and provides immediate fluorescent response after hybridization to complementary nucleic acid analytes, thus offering easy and instant detection of specific DNA and RNA [78].

The choice of chromophore is crucially important for widespread practical application of aptamer probes to live cell imaging. Although malachite green has been successfully applied to the binary aptamer probe, it remains uncertain whether malachite green is the best choice for intracellular imaging. It is worth mentioning that malachite green very efficiently generates singlet oxygen upon irradiation, and is used for targeted damage of mRNA constructs [75], and may therefore also lead to undesirable consequences for the behavior of cells during the imaging process. Therefore, an alternative chromophore that is less toxic and better suited for live cell imaging is desirable.

Therefore, we isolated an RNA aptamer against the cyanine dye Cy3 (Cy3apt), a widely used, permeable and nontoxic fluorophore [82]. The parental Cy3apt was

83 nts long and was shortened to 49 nts long with increased affinity to Cy3, achieved by multiple base changes (Fig. 6a). The affinity of Cy3apt to Cy3 was examined by SPR using a Cy3-immobilized sensor chip injected with different concentrations of Cy3apt. The SPR signal reached a plateau immediately upon injection of Cy3apt RNA, and plateau levels increased in proportion to the amount of RNA injected, followed by rapid dissociation when the injection stopped (Fig. 6b). Interestingly, the fluorescence intensity of free Cy3 at 580 nm (50 nm bandpass) increased in proportion to the Cy3apt RNA added, with no detectable change in the emission spectra [82].

The shortened derivative of Cy3apt is composed of two separate hairpin modules (Fig. 6a). Although each of these domains has no affinity to Cy3 separately, they exhibit affinity to Cy3 upon being properly arranged in a tertiary configuration.

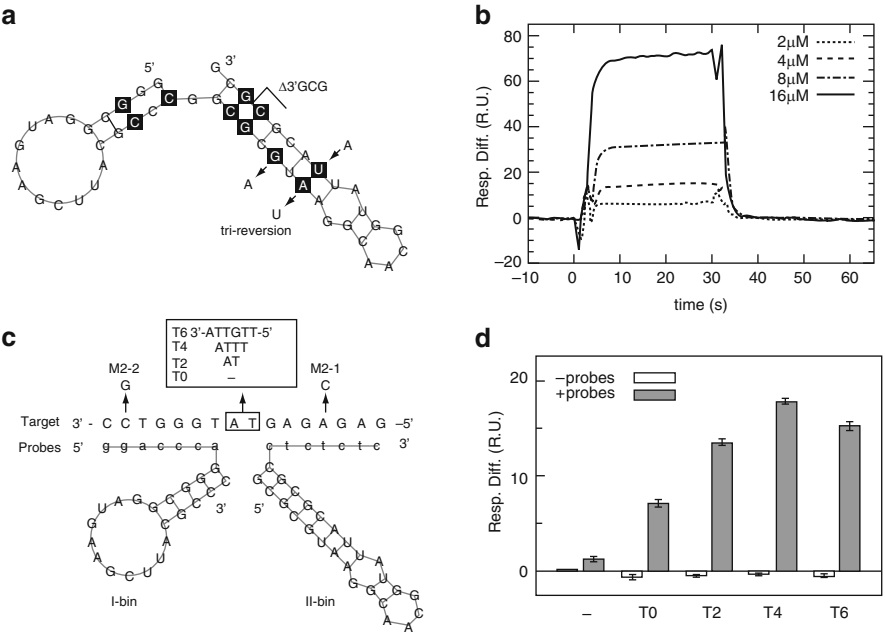

Fig. 6 Binary Cy3 aptamer probe composed of folded modules. (**a**) Optimized structure of Cy3apt. Ten nucleotides shown in *black squares* represent substitutions from the original Cy3apt sequence to optimize affinity to Cy3. *Δ3′GCG* denotes a three-base deletion on the 3′ end. *Tri-reversion* indicates three bases that are reverted to that of the original. (**b**) SPR sensorgrams of Cy3apt binding to Cy3 immobilized on the sensor chip. The indicated concentrations of RNAs were injected at time 0 for 30 s at a flow rate of 10 mL/min. (**c**) Binary Cy3 aptamer probe to detect target oligonucleotides. Schematic representation of the target oligonucleotide and the binary aptamer probes (*I-bin* and *II-bin*). The target oligonucleotide *T2* sequence is shown and the variable linker sequences are boxed. *M2-1* and *M2-2* are single nucleotide mismatches introduced into T2. Target binding sequences of the binary probe are depicted in *lowercase letters*. (**d**) Detection of target oligonucleotides using the binary probe as SPR signals. Target oligonucleotides (10 mM) with (*shaded*) or without the binary probe (16 mM) were subjected to the SPR analysis

Each domain of the Cy3apt was separately used to construct a binary Cy3 aptamer probe. A heptanucleotide, corresponding to each half of a complementary sequence of a 14 nucleotide target sequence (T0 in Fig. 6c) was appended onto the 5′ terminus of one domain and the 3′ terminus of the other domain as a target-binding arm (I-bin and II-bin in Fig. 6c). In contrast to the preceding studies [78], both of our binary probe elements fold into stem–loop structures and have no single-stranded extension except for the appended flanking sequences required for target recognition. The Cy3-binding activity of the binary probe was analyzed by SPR. As shown in Fig. 6d, the designed Cy3 aptamer probe alone did not bind to Cy3 in the absence of target oligonucleotides. When the target oligonucleotides were present, the aptamer probe bound to Cy3 (Fig. 6d). The binding efficiency varied depending on the nucleotide length of the central linker sequences in the target oligonucleotides, T2, T4 and T6 (see Fig. 6d). The best affinity in these experiments was observed with T4, which contains a tetranucleotide insert, suggesting that the orientation of the two probe elements is important in regenerating the tertiary structure so that it is capable of binding to Cy3. When a single mismatch was introduced into each recognition site of the T2 target sequence (M2-1 and M2-2 in Fig. 6c), the binary probe did not bind the target sequence (Fig. 6d), demonstrating single nucleotide discrimination. This binary probe consists of two folded modules, hence referred to as a folded binary probe [82].

3.3 Anti-Midkine Aptamer to Therapeutics

Midkine (MK) is a heparin-binding growth factor and exerts pleiotropic effects, including cell proliferation, cell migration, angiogenesis, and fibrinolysis in a variety of tissues [83]. MK also plays important roles in the induction of oncogenesis, inflammation, and tissue repair. The overexpression of MK has been observed in a number of malignant tumors, Hodgkin's disease, and brain tumors [83]. On the other hand, MK-deficient mice are reportedly resistant to ischemic renal injury [84] and neointima formation in atherosclerosis [85]. A recent study proposed that MK deficiency suppresses the development of a rheumatoid arthritis model by preventing inflammatory leukocyte migration and osteoclast differentiation [86]. Furthermore, MK expression is upregulated in the spinal cord during the induction and progression phase of experimental autoimmune encephalomyelitis (EAE) [87].

EAE is a mouse model of multiple sclerosis (MS), an autoimmune disease characterized by inflammatory demyelination in the central nervous system (CNS) [88]. Although MS and EAE have been described as T-helper type 1 (T_H1) cell-mediated autoimmune diseases, various types of immune cells and soluble mediators contribute to the complex mechanism underlying the onset and progression of MS, which is characterized by autoreactive T cell infiltration and the activation of microglia, the resident antigen-presenting cells in the CNS. In particular, $CD4^+$ $CD25^+$ regulatory T (T_{reg}) cells have received a great deal of attention as negative regulators of MS pathogenesis [89–92]. T_{reg} cells regulate peripheral

tolerance and autoimmunity, and abnormalities in T_{reg} cell function may contribute to the development of autoimmune diseases [93–95]. Thus, expansion of the T_{reg} cell population could prevent autoimmune attacks, such as gastritis, oophoritis, thyroiditis, inflammatory bowel disease, and MS [90–92, 96, 97].

Suzumura and colleagues found that MK-deficient mice are resistant to myelin oligodendrocyte glycoprotein (MOG)-induced EAE due to an expansion of the T_{reg} cell population in the peripheral lymph nodes, followed by a decrease in the numbers of autoreactive T_H1 and T_H17 cells [98]. We isolated RNA aptamers against MK to develop a potent therapeutic strategy against autoimmune diseases, including MS. Reflecting a protein-like affinity to heparin, several high-affinity anti-MK aptamers were selected (three representative M-fold structures are shown in Fig. 7a).[1] These parental sequences were then shortened and optimized further for in vitro and in vivo efficacy tests. One such derivative, 49 nts in length, showed a K_d of 0.9 nM, as estimated by SPR analysis (Fig. 7b) [98]. This aptamer and a shortened derivative of 38 nts were stabilized with modifications at the 2′ position of ribose, conjugated at the 5′ end with cholesterol, conjugated at the 3′ end with inverted deoxythymidine (dT), and examined for in vivo efficacy [98, 99].

To investigate the efficacy of the modified 38-nucletoide aptamer MKapt in the pathogenesis of EAE, we immunized C57BL/6 mice with myelin oligodendrocyte glycoprotein (MOG) peptide in complete Freund's adjuvant, and MKapt was administered i.p. (0 and 1 mg/kg dosages) every other day. Chronic progressive EAE was observed in the immunized, untreated mice (control, $n = 5$) at day 14, and severe EAE by day 20 (Fig. 7c). In contrast, the appearance of EAE was significantly delayed in mice administrated with 1 mg/kg MKapt, with symptoms markedly reduced (Fig. 7c). Mice were sacrificed at day 28 and subjected to histological analysis. Consistent with clinical signs, typical foci of mononuclear cell (MNC) infiltration and demyelination were observed in the white matter of the spinal cord of untreated mice, but these signs were not observed in most mice administered with 1 mg/kg MKapt. Thus, a clear correlation was found between clinical and pathological features of EAE between untreated and MKapt-treated mice. Moreover, the administration of MKapt induced expansion of the T_{reg} cell population [98]. These findings suggest that MK is a suppressor of T_{reg} cells, and that blockade of MK by RNA aptamer may be a potent therapeutic strategy against autoimmune diseases, including MS.

4 Concluding Remarks

In this laboratory, RNA aptamers have been selected against a variety of human proteins and a summary of these studies is presented in this review. Although many properties of the selected aptamers are similar to those of antibodies, the aptamers

[1] http://www.wipo.int/pctdb/en/ia.jsp?IA=JP2007072099.

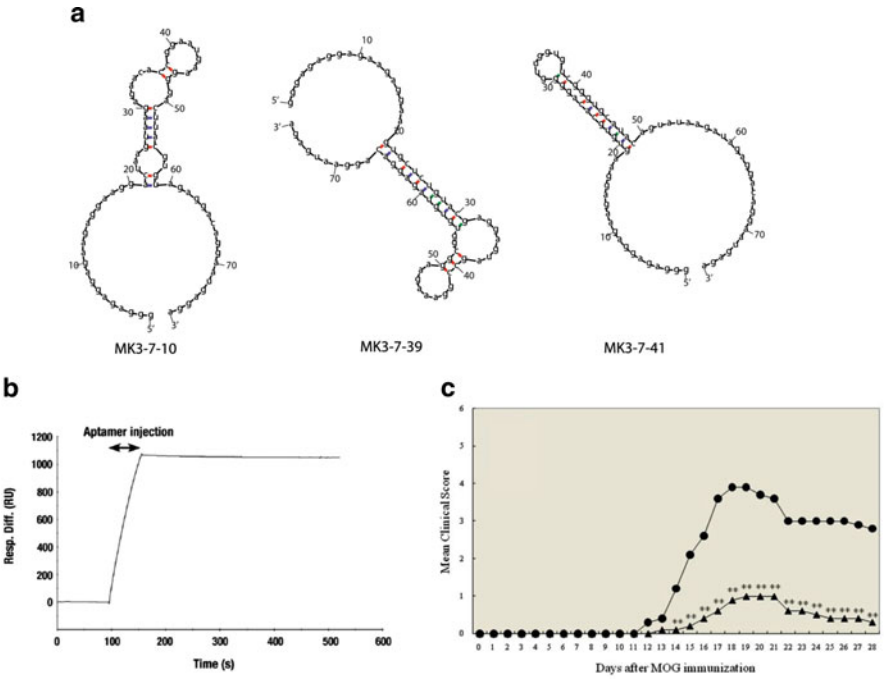

Fig. 7 Anti-MK aptamer and its therapeutic potential. (**a**) M-fold predicted secondary structures of three representative MKapt sequences. (**b**) Sensorgram of MKapt (49-nucleotide derivative) binding MK. MK was immobilized on a CM4 sensor chip and the RNA aptamers were injected for the indicated period. The K_d was estimated to be 0.9 nM using global fitting curves (1:1 Langmuir binding) with four different RNA concentrations (0.05, 0.1, 0.2, and 0.4 mM). (**c**) Clinical scores for wild-type EAE mice administered phosphate buffered saline (*circles*) ($n = 5$) or 1 mg/kg ($n = 5$) MKapt (38-nucleotide derivative) (*triangles*) after EAE onset (14 days after immunization). ** $P < 0.01$ for 1 mg/kg MKapt versus phosphate buffered saline (Mann–Whitney's U test or Dunnett test)

also exhibit superior features. Selected aptamers occasionally had a K_d on the picomolar scale, an affinity which is a hundred times stronger than normal antibody–antigen interactions. Molecular and biochemical analyses revealed that certain aptamers need to be >50 nts long for specific and high-affinity binding to their target proteins. Therefore, it might be argued that the high affinity of RNA aptamers to proteins that do not have RNA recognition motifs or an intrinsic, strong affinity to RNA is achieved through the capture of the protein's global conformation, as shown for the eIF4A aptamer (see Fig. 2b). In contrast, the anti-hFc1 aptamer achieved strong and specific binding to hFc1 mainly by van der Waals contacts and hydrogen bonds, rather than via the electrostatic forces between the negatively charged phosphate backbone and the positively charged protein surface that characterize most protein–nucleic acid interactions. These findings demonstrate that RNA has a high potential to form a vast set of tertiary structures, and we would like to refer to this property as "RNA plasticity." This conformational

plasticity and selectivity can be achieved by multiple interactions, which are applicable to many protein targets of low or no affinity to nucleic acids. These results provide us with a solid and promising basis for steps to create novel RNA molecules with distinct structures and with therapeutic potential superior to that of antibodies.

To date, no translation factor-specific therapy has been developed. Nevertheless, recent data [100] demonstrates the first feasible employment of eIF4E-specific therapy for the treatment of human malignancies. The data illustrate that systemic administration of second-generation eIF4E-antisense oligonucleotides can effectively target eIF4E for destruction in xenograft tumor tissues, significantly suppressing tumor growth and affecting angiogenesis. Moreover, even with the profound reduction of eIF4E expression in the liver, mice showed no adverse effects. These data reinforce the notion that targeting eIF4E will selectively affect tumor tissues compared with normal tissues and provide the foundation for advancing eIF4E-targeting RNA molecules to clinical trials for the treatment of human malignancies.

Therapeutic antibodies are rapidly developing worldwide. In 2009, 28 antibody-based therapeutics were approved by the FDA, and the antibody therapeutic market is expected to reach in excess of 30 billion dollars in 2009. Moving forward, aptamer therapeutics are not at a disadvantage. Several characteristics of aptamers give them superior potential to therapeutic antibodies, including increased binding affinity, in vitro manipulation of activity and/or stability, less immunogenicity or toxicity, and scalable chemical production. In contrast to costly cell-based production of antibodies, the production costs of RNA aptamers will also be greatly reduced with the development of oligonucleotide-based therapies. Therefore, not only from a therapeutic standpoint, but also from healthcare economics, RNA aptamers offer a beneficial therapeutic approach for the treatment of diseases.

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Development and Clinical Applications of Nucleic Acid Therapeutics

Veenu Aishwarya, Anna Kalota, and Alan M. Gewirtz

Abstract In the past decade, our new understanding of post-transcriptional gene silencing (PTGS) has opened up new vistas that can help us to decipher the function of genes and thus to unravel the basic mechanistic principles and genetic networks in biology. This enormous potential of PTGS to selectively turn off genes either by antisense or RNA interference pathways has made a huge impact on research in basic science. Further, we can apply this knowledge and new set of rules in biomedical research for the development of more potent therapeutics. With a plethora of advantages of PTGS, there are still certain obstacles that need to be addressed to enable translation of this technology to the clinic. This article will focus on a brief history of the discovery and evolution of PTGS and its potential as a technology. We will also discuss the importance of target availability and some strategies for identification of the target sequence. Further, we will give an overview of some past experiences in the development of this technology in the clinic for cancer therapy, with a recent update on clinical development and trials on nucleic acid therapeutics.

Keywords Antisense · Cancer therapy · Clinical trials · Oligodeoxynucleotides · Post-transcriptional gene silencing · RNA interference · RNase H · siRNA

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Dedicated to the memory of our mentor and a visionary – Dr. Alan M. Gewirtz.

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1 Introduction

Health care has seen a tremendous change in invention and application in the past decades. This evolution has been rapid and has seen the emergence and disappearance of many classes of therapeutics. The huge investment in time and cost of development is the major reason why there is a need to look for better, revised, and new alternatives in technology to ensure the development of more efficient drugs for treatment of disease. New strategies for the development of nucleic acid therapeutics are showing considerable progress as compared to a decade ago. To date there is only one drug based on this antisense technology that has been approved by the FDA: Fomivirsen (brand name Vitravene) for treating cytomegalovirus retinitis. However, more drugs are being evaluated in different phases of clinical trials and we are in the process of developing strategies relating to target site accessibility, chemistry, and delivery of these molecules. Scientists believe that nucleic acid-based therapies are a promising tool and can serve as a cure for many diseases.

In the past few years, the drug industry has seen the emergence of a new field of scientific speculation involving gene silencing using different forms of nucleic acids. Post-transcriptional gene silencing (PTGS) is an evolutionary conserved phenomenon that can occur by many mechanisms in the cell. One of the most widely studied processes is RNA interference (RNAi), in which small double-stranded RNA (dsRNA) pairs initially with specialized proteins to form RNA-induced silencing complex (RISC), which further induces sequence-specific cleavage of a target mRNA molecule [1, 2]. Gene silencing can also be induced by a completely different pathway in which an exogenous antisense DNA can specifically bind to target mRNA and initiate cleavage by another mechanism facilitated by ribonuclease H (RNase H; an enzyme that hydrolyses the RNA strand of an RNA/DNA duplex) [3, 4]. The simplicity of these actions should not be taken for granted as these mechanisms have their own set of complex pathways that research laboratories across the globe are still trying to comprehend. This fact can be appreciated by the explosion in the number of research articles in the area, indicating the exponential increase in the efforts to understand the underlying mechanisms involved in gene regulation, especially by these two mechanisms. As with any biological mechanism, the more we try to comprehend PTGS, the more

complex and interesting it becomes, with the discovery and inclusion of new biological players every subsequent year. This also imposes the development of smart innovative strategies using advanced and exaggerated means to ameliorate our smattering of understanding in the area.

One of the major reasons why an in-depth understanding of this particular discipline of research is of utmost importance, is that it allows us to indulge in both basic and applied sciences. PTGS can very well be appreciated as a part of basic science by exploiting its ability for sequence-specific cleavage of mRNA and thus decipher the functions of specific genes involved in various biological networks [5]. Moreover, and more importantly, this aspect can also be applied to target genes involved in various anomalies and thus can be used as a therapeutic tool [6]. It is therefore essential that we have an astute understanding of this field so that it can be used efficiently in any form.

Development of antisense oligonucleotides (AS-ON) and small RNA-based therapeutics comes with an entire package of veiled mechanistic issues. There is a need to address these basic but paramount problems, including a more comprehensive understanding of the pathways and mechanisms, precisely at the structural level, of how these small RNAs interact with the proteins that help them to target and cleave the target mRNA [7–12]. A relatively less studied issue involves target site accessibility, which may be affected by target mRNA folding and/or local mRNA binding proteins bound to the targeted mRNA molecules [13–17]. These issues are followed by secondary and equally important problems like the development of AS-ONs and short interfering RNA (siRNA) molecules that are compatible with the cell's microenvironment and protein machinery, especially by introducing chemical modifications [18, 19]. A final problem is the delivery of these molecules to the desired cells [20–23]. Each aspect in itself has a number of intricate complexities and all are equally significant in achieving the ultimate goal of efficient silencing of the gene in question. Rational selection of mRNA target sites, intracellular stability, and delivery into cells of interest are among the problems that need to be resolved.

2 Brief History of Post-Transcriptional Gene Silencing

2.1 Antisense Oligodeoxynucleotides

Belikova et al. first envisioned the use of antisense oligodeoxynucleotides (AS-ODNs), which was later demonstrated for the first time in vitro by Paterson et al. ten years later in 1977 utilizing the rabbit beta globin clone PbetaG1 [24, 25]. AS-ODNs can be considered as the simplest type of nucleic acid molecules and were the first agents to be used to knockdown gene expression in a sequence-specific manner. AS-ODNs inhibit gene expression by complementary base pairing to the target mRNA. Binding of the ODN to the target mRNA facilitates gene

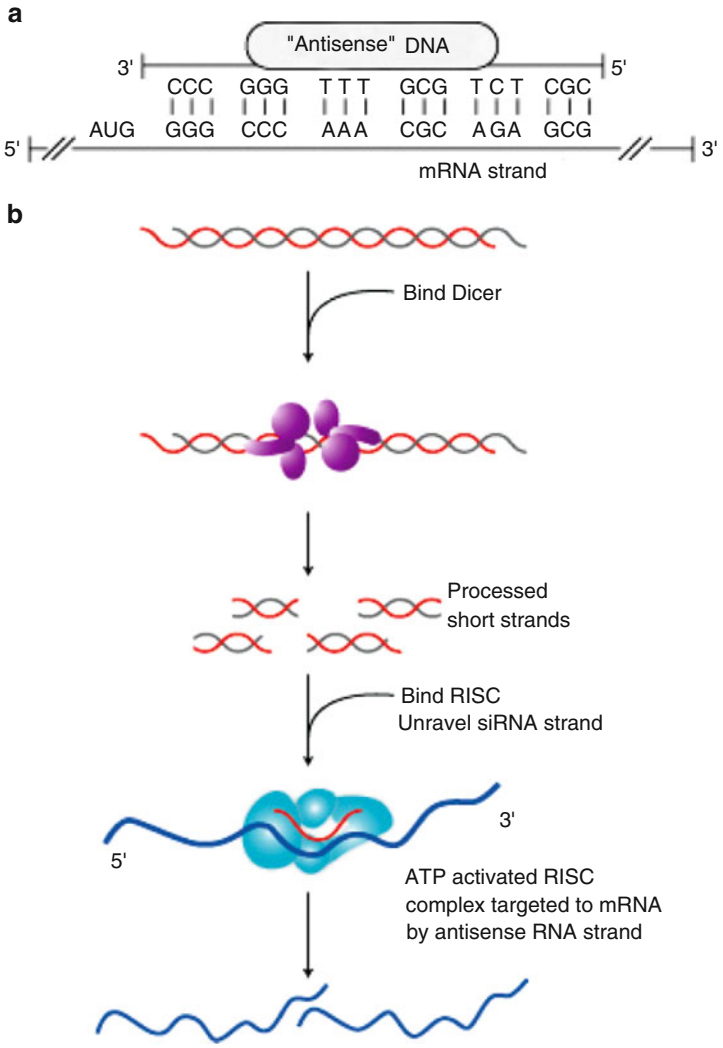

Fig. 1 Strategies for inhibiting translation with single-stranded nucleic acid molecules. All molecules are shown hybridized to their mRNA target. (**a**) Simple oligodeoxynucleotide. Cleavage of mRNA depends on RNase H. (**b**) Mechanisms underlying RNAi

repression and can be induced via two different mechanisms. The first mechanism is via RNase H-mediated cleavage of RNase H-dependent ONs. Hybridization of AS-ODN to the target mRNA can initiate RNase H activity, which cleaves the 3′-O-P-bond of RNA in a DNA/RNA duplex to produce 3′-hydroxyl- and 5′-phosphate-terminated products (Fig. 1a). The second mechanism involves ONs that hybridize to the mRNA and cause a steric hindrance that facilitates the termination of translation by blocking the ribosomes [26, 27]. This can also inhibit

the progression of the splicing machinery. However, most studies have exploited the RNase H-mediated pathway of cleavage for ODN-mediated silencing.

AS-ODNs have been widely used in different systems by various research groups. Zamecnik and Stephenson first reported the inhibition of Rous sarcoma virus replication and cell transformation using an ODN that was complementary to 13 nucleotides of the 3′- and 5′-reiterated terminal sequences of Rous sarcoma virus 35S RNA. Their results indicated interference caused by these ODN sequences in the pathway(s) involved in viral production and cell transformation [28]. In a seminal paper, Gewirtz and Calabretta used AS-ODNs to show that the c-myb protooncogene (see Sect. 5.2) plays a crucial role in regulating normal human hematopoiesis [5]. A year later, Anfossi et al. reported the use of an ODN complementary to an 18 bp sequence of c-myb mRNA and observed that the nuclear protein encoded by the c-myb protooncogene is required for maintenance of proliferation in certain leukemia cell lines [29]. These and more studies using AS-ODNs compel us to appreciate the evolving applications of these molecules [30].

ODNs have been used both in unmodified and modified forms. The latter are more likely to dominate because unmodified forms are rapidly degraded by the intracellular endonucleases and exonucleases, and are less stable [3, 31–33]. Optimizing the chemistry of ODNs has been the subject of much debate and speculation. It has been suggested that changes in the sugar moiety may influence RNase H activation. Moreover, modifications in the backbone may increase the activity of RNase H [34]. Methyphosphonates, which were the first chemically synthesized and non-charged ONs, were soon replaced in studies by phosphorotioates. Although having the advantage of being biologically stable, methyphosphonates have reduced cellular uptake because of the absence of charge and are not able to induce RNase H activity [35]. Phosphorothioates are preferred because they are easy to synthesize and, more importantly, impart greater nuclease stability. However, the problem of nonspecificity and their propensity to bind to proteins, especially those that interact with polyanions [36–38], led to development of the chimeric ONs [39, 40]. Further modifications facilitated development of second generation molecules that involved alkyl modifications by introduction of methy or methoxy-ethyl groups at the 2′ oxygen of the ribose.

Recently, more advanced analogs have been designed. Also known as third generation ONs, the modifications include the substitution of functional groups of the ribose by amino or fluoro groups. Kalota et al. proposed 2′-deoxy-2′-fluoro-β-D-arabinonucleic acid (2′F-ANA) sugar modifications and claimed the products to be nuclease resistant, to form a stable ON/RNA duplex, and to be RNase H compliant. Moreover, these modifications were helpful in maintaining high intracellular concentrations of ONs for a longer period of time. The direct effect of these modifications was tested by targeting mRNA of the human c-myb protooncogene. Chemically modified ODNs were found to be 90% more effective than AS-ODNs containing unmodified nucleosides. Moreover, the longevity of the effect was appreciated and effective silencing observed, even after 4 days of dose administration [41, 42]. Further, analogs with peptide bonds in place of the ribose-phosphate

backbone have also been specifically designed to induce RNase H activity and to have greater and more specific binding capacity to the target RNA.

2.2 siRNAs and RNA Interference

The second group of players in the race towards sequence-targeted therapeutics are short duplexes of RNA molecules known to trigger a potent and highly specific gene-silencing phenomenon called RNA interference (RNAi). It was first demonstrated by Fire and colleagues in the nematode *Caenorhabditis elegans* [43]. Unlike the veiled underlying mechanisms of RNase H-mediated cleavage, RNAi has been more extensively characterized. RNAi is initiated by the conversion of long dsRNA molecules into smaller 21–23 nuncleotide (nt) dsRNA molecules known as siRNAs (Fig. 1b). The key player in this process is a RNase III-type enzyme known as Dicer. RNase III enzymes are known to have a DEXD/H ATPase domain, a DUF283 domain, a PAZ domain, two RNase III domains and a dsRNA binding domain. Dicer has been extensively studied in various systems. It was reported that the nematode *C. elegans* and mammals have a single Dicer that catalyzes production of both siRNAs and microRNAs (miRNAs) [44–47]. Interestingly it was also observed that *Drosophila* has two different types of Dicers for individual processing of these molecules [48]. Dicer processing creates RNAs with 2-nt overhangs at the 3′ ends and a phosphate group at the 5′ ends, forming the siRNA duplex [49, 50]. The sense and antisense strands of this duplex are named with respect to the orientation of the target mRNA. The sense strand that is later degraded is the passenger strand, while the antisense strand or the guide strand acts as the template.

The next step involves the formation of RISC loading complex (RLC). This complex contains a Dicer and dsRNA binding protein (R2D2) hetrodimer. Further, this complex orients the RNA duplex in appropriate orientation for the subsequent RISC assembly. Thermodynamic stability of the duplex determines which stand stays and which is released in the complex. The stability of the 5′ ends of the two stands play an important role in this selection of the guide strand. The strand with its 5′ end at the less thermodynamically stable end of the duplex is selected as the guide strand [51, 52]. In human cells, Argonaute 2 (AGO2) aids in guide strand selection by slicing the passenger stand, and its subsequent release marks the conversion of RLC to RISC. The guide strand-laden RISC uses this stand for the sequence-specific silencing of the mRNAs that contain its complementary sequence. The specificity of this mechanism can be appreciated by the fact that the cleavage takes place between 10 and 11 nucleotides upstream of the 5′ end of the guide strand.

As mentioned before, siRNAs have been widely used in investigating the basic mechanisms of biology. There are several studies involving gene knockdown aimed at answering complicated biological problems. With the availability of the sequencing data of genes of various genomes, the applicability of RNAi widens day by day.

There have been studies using RNAi to understand DNA damage processing, genome stability, and mutagenesis. It has been widely used to comprehend gene function in vitro and in vivo by reverse genetics, in which the function of the target gene can be determined by its cleavage and/or disruption. The gene knockdown capacity of siRNAs in non-dividing and terminally differentiated cells such as neurons or macrophages makes them an ideal tool for study in cells that are difficult to transfect. The ability of siRNAs to persist for a long time, even for weeks, in some systems makes them superior to pre-existing conventional tools. Functional and genome-wide RNAi analysis have been performed in various studies in *C. elegans, Drosophila*, and mammalian cells [53–56].

The therapeutic potential of RNAi has also started to emerge in the past few years. The major factors that make siRNA an ideal tool include the specificity of action, ease of synthesis, low cost of production, and stability. Development of siRNA-based therapeutic agents has long started in almost every area of medicine and is well covered in other seminal reviews [57–68].

The efficiency of any AS-ON or siRNA largely depends on the accessibility of the target mRNA, which can be a major problem due to the formation of secondary structures or due to the binding of the local proteins in the cell. The various phases of development of these gene-silencing systems lead to different levels of problems related to specificity, stability, and delivery. We will now discuss the preliminary approaches and developments related to mRNA target accessibility, which may be hampered either by formation of secondary structures or by the binding of proteins.

3 Strategies for the Identification of Target Sequences and Structures

One of the major obstacles, apart from delivery, in employing AS-ODNs or siRNAs for PTGS is the accessibility of the target sequence within the target mRNA. This is influenced by the formation of higher-order structures and the occupation of the target sites by RNA binding proteins. Numerous computer-generated algorithms have been developed to simulate the RNA folding and to identify the formation of secondary and tertiary structures by considering the thermodynamics, kinetics, salt concentrations, temperature etc. Although these algorithms are helpful in predicting the secondary and tertiary structures, they are still not very efficient. Further, the still-unknown RNA binding proteins make the problem more complicated because these proteins are both sequence- and/or structure-specific and most have uncharacterized domains.

Many strategies have been proposed in order to overcome these problems and to identify a perfect target site, primarily the “oligo-walk” method. Initially demonstrated by Bacon and Wickstrom, in this strategy a series of AS-ONs complementary to various regions of the target mRNA were developed and tested for their capacity to inhibit mRNA expression [69]. The major disadvantages of this

strategy are its cost and its time-consuming aspect, as many sequences have to be tested to find a potential molecule.

Chimeric ODN libraries and RNase H were also employed to identify regions on the target mRNA that are available for hybridization [70]. Ho et al. described another approach for mapping the target sites that involves the development of a semi-random ON library to probe the target mRNA. This involves the cleavage induced by RNase H, which identifies the DNA/RNA hybrid, when the complementary sequence of the library hybridizes with the accessible region of RNA. This was followed by gel sequencing to visualize the cleavage product [71]. There were also reports of the use of matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) spectrometry to analyze the cleaved fragments. Further, Allawi et al. used an ON library in conjunction with a reverse transcription assay to identify accessible sites of the target mRNA [72]. The random ONs were used to prime complementary DNA (cDNA) synthesis in the presence of reverse transcriptase in order to generate a series of cDNA fragments. The initiation of cDNA synthesis occurs only at RNA sites that are available for ODN binding. These products were then analyzed by gel sequencing. Another method proposed for selecting effective target sites exploited arrays of ONs complementary to the target mRNA. In this method, potential sites for antisense are identified as ODNs that form stable hybrids with the target mRNA [73, 74].

In an attempt to prevent the formation of higher-order structures of RNA, a different strategy was evaluated. Pseudo-complementary bases 2-aminoadenine (nA) and 2-thiouracil (sU) and the destabilizing base 7-deazaguanine (cG) were introduced. Due to steric hindrance, nA and sU cannot from a stable base pair and are thus pseudo-complementary; however, regular T/U and A complements allow pairing with ONs. The practical application of this strategy to ON-based hybridization assays will require engineering of RNA polymerase for more efficient utilization of pseudo-complementary nucleoside triphosphates [75, 76].

Another strategy includes the development of a new RNA mapping strategy combining computational and experimental approaches [77, 78]. We developed fluorescent self-quenching reporter molecules (SQRM) that are used to identify regions within the target mRNA available for hybridization with AS-ONs (Fig. 2). SQRMs are typically DNA hairpin molecules of 20–30 bases with 4–5 base complementary ends. The 5′ end of the sequence is linked to a fluorophore (fluorescein) and 3′-end to the quencher (DABCYL). In the absence of the target mRNA, the SQRM folds such that the fluorescein is quenched by DABCYL and no signal is detected. Once the SQRM is mixed with the target and hybridization takes place, the fluorophore and quencher are separated and the fluorescence signal can be detected. In order to design SQRM sequences, a computer algorithm was developed to scan the sequence of the gene of interest for inverted repeats of 4–5 bases separated by an inverting sequence of 18–20 bases. Antisense sequences selected based on this strategy were successfully tested in cell culture experiments.

A modification in this strategy was employed to better understand how efficient commonly used computer programs are at determining RNA folding. In this study, the presence of the stem–loop structures was shown to be of little value for SQRMs

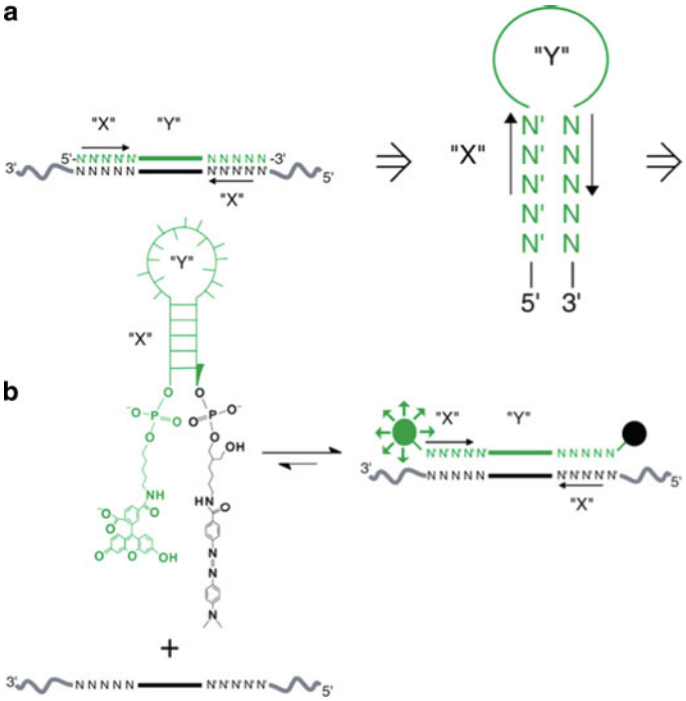

Fig. 2 Design and reaction of self-quenching reporter molecules (SQRM). (**a**) To exploit the traditional stem–loop structure of the SQRMs, a computer algorithm searches an entire sequence of mRNA for complementary sequences of a desired length (*X*, stems) that are separated by a proscribed distance (*Y*, loop). (**b**) The complementary sequences are synthesized as SQRM possessing 5′-fluorescein and 3′ DABCYL groups. In the absence of target, quenching of fluorescence occurs. Once hybridization of the loop sequence to a complementary target takes place, the moieties are separated and fluorescence can be detected [78]. Image reprinted by the permission from Oxford University Press

to function. Two types of SQRMs were developed and were classified as type I or type II. Type I molecules had a stem–loop structure, whereas type II molecules were devoid of the stem. The entropies, enthalpies, and the melting temperatures of folding of SQRMs were determined. The results indicated a surprising difference in the structures of these molecule variants and we observed that that type I SQRMs were unstructured at 37 °C whereas some of the stem-less type II SQRMs were structured. This data failed to correlate with the data generated for the commonly used RNA folding tool "mfold." This may be due to the fact that mfold does not take into consideration the exact experimental solvent conditions to calculate its thermodynamic values. Moreover, other factors that are present in the cell's microenvironment (e.g., the presence of binding proteins) cannot be taken into consideration by mfold, thus we see a discrepancy in the results [79, 80]. For better knowledge of the target sites, there is a need to develop more robust experimental techniques to better understand RNA structure and how it behaves in a cell's microenvironment.

4 Understanding Target Site Accessibility: Experimental Approaches

In the last few years there have been interesting reports investigating the problem related to target site accessibility. Here, we discuss a few interesting observations that have helped our understanding of this aspect. In a study by Brown et al., the effect of enhanced target site accessibility on the catalysis of activated human RNA-induced silencing complex (RISC*) was examined [14]. HIV-1 transactivation responsive element (TAR), which has a well-characterized stem–loop structure, was used in this study and it was observed that increased access to the target site enhanced the efficiency of siRNA-programmed RISC* cleavage of target RNA. These observations reiterate the fact that the inclination of RNA to form secondary and/or tertiary structures may obstruct sites of hybridization and thus hamper physical access to the target site sequence. In another interesting example, Ameres et al. showed that RISC is unable to unfold structured RNA and that accessibility of the target sites have a direct correlation with the efficiency of cleavage [13]. The data showed that the structure of target mRNAs effect the RISC-mediated target recognition, even with similar accessibility to the target site. They also showed that the presence of a single-stranded region upstream of the target site had a better cleavage efficiency than when the single-stranded region was replaced by a stem–loop structure, thereby suggesting that single-stranded regions are preferred within target RNAs and can enhance RISC cleavage [13].

Another study targeted enhanced green fluorescent protein to demonstrate that the accessibility of certain local target structures on the mRNA is an important determinant in the gene silencing ability of siRNAs [81]. A computer algorithm for minimal siRNA selection was selected and was used for the categorization of these molecules based on the target transcripts' minimum free energy structures predicted by the algorithm. Results from the transfection experiments showed that mRNAs predicted to have unpaired 5′ and 3′ ends to which the siRNAs were targeted showed a greater degree of gene silencing than targets having other types of secondary structures. This effect was not related to the structure of the siRNA guide strand. These studies were in accordance with the previously published reports showing higher silencing efficiency of the mRNA targets having unpaired 5′ or 3′ ends than targets unpaired in the middle region. These observations elicit the minimal requirements for hybridization between mRNA and the guide strand of siRNA and emphasize the importance of the selection of both guide strand and mRNA when choosing siRNA candidates.

Westerhout and Berkhout studied the structure-based resistance that can be imposed by a target, by targeting a perfect hairpin. For the comparison, a mutation was made in the tight structure that destabilized it and exposed the target sequence. An inverse correlation between the efficiency of RNAi within a specific thermodynamic stability range and target hairpin stability was observed. Improved binding of the siRNA to the destabilized target RNA hairpins was

thought to be the cause of increased RNAi efficiency. Another significant aspect of the study was an in vivo and in vitro comparison of the accessible regions, especially the 3′ end of the target, which indicated that the 3′ end of the target is an important factor for RNAi-mediated cleavage. Interestingly, the result was not reproducible in vitro, indicating the important role of RISC components in in vivo systems. These studies were in harmony with the previous observations showing that the TAR hairpin of the HIC-1 genome is an unsuitable target because of its tight structure. This illustrates that the target structure is also an important factor when selecting a suitable target sequence [82].

Another group addressed the problem of identification of the relationship between RNAi-mediated cleavage and the sequence and/or structure of the target, and attempted to explore the tight structure of the TAR element within the target RNA, which adopts a very stable secondary structure. The results suggested that the tight stem–loop structure of TAR had an effect on capability of siRNAs to mediate cleavage. Other effects related to the position of the binding of the Tat protein to the TAR element, and the location of the target within a translated or a noncoding region did not have substantial effects on RNAi. However, the orientation of the target sequence had a substantial effect, indicating that specific nucleotides at certain positions are favorable for this process. To check the location of the target site, 47 different sites within 47 plasmids were checked under identical conditions and the findings led to the conclusion that the location is not the major determinant of siRNA activity but that the target sequence itself plays a major role. This also leads to the speculation that another strategy for developing siRNA molecules could be to include preferred nucleotides at specific positions. However, this may vary in different systems and a consensus is yet to be reached [83].

To understand the constraints of mRNA structure and in an attempt to bridge some gaps in these studies, a comprehensive approach was undertaken by Rudnick et al. [15]. In this study, the authors compared the activity of RNase H- and RISC-induced mRNA cleavage. This was important for comparing their individual efficiencies. The effect of imposing additional structures upstream and downstream of the target site and the effect of additional structures within the target site in AS-ON- and siRNA-mediated mRNA cleavage was also studied. Their observations suggested that both AS-ON- and siRNA-mediated cleavage complexes have the ability to deal with significant levels of target-site structure over time. Interestingly, it was also speculated that cleavage proceeds, if the target site is uninvolved in a complex structure formation and thus there is access to the site. As mentioned before, to address and better understand the effect of RNA binding protein on antisense mRNA cleavage, a significant study was performed using a well-defined RNA binding protein, αCP. It was observed that αCP effectively blocked AS-ON -mediated cleavage of the RNA binding site compared with cleavage of the site in the absence of αCP. This shows the possibility of the formation of higher order structures by RNA and RNA-bound proteins, which may play an important role in determining the efficacy of AS-ON-directed PTGS [15]. RNA structure still remains a “black box” and there is a need to develop strategies to identify “working target” sites for hybridization. However, a combination of

computer algorithms and, more importantly, novel experimental methodologies can help gain a better understanding of RNA structure and how it reacts in the microenvironment of cells in the presence of known and unknown proteins, and thus help to solve these complex issues.

5 Past Experiences in Cancer Therapy

Cancers, in particular, are attractive candidates for antisense therapy. The problem with conventional therapies is that they are highly toxic. Since antisense strategies are directed against specific genes that are aberrantly expressed in diseased cells, it is expected that this approach will cause fewer and less serious side effects. Several genes implicated in many cancers have been intensively studied as potential targets for antisense therapy, and these studies are discussed below.

5.1 B-Cell Lymphoma Protein 2

B-cell lymphoma protein 2 (Bcl-2) is an apoptosis-regulating oncogene and its overexpression has been reported in most follicular non-Hodgkin's lymphomas (NHL), other lymphomas and leukemias, as well in other kinds of cancers like lung, breast, colorectal, gastric, prostate, renal, and neuroblastoma [4]. This overexpression is usually associated with an aggressive malignant clone characterized by resistance to standard chemotherapeutic agents, early relapse, and poor survival outcome. Laboratory studies have shown that exposing cells to the ONs directed against Bcl-2 will specifically and significantly decrease mRNA and protein expression [84]. For all of these reasons, there is a great deal of interest in targeting Bcl-2 for therapeutic purposes. The results of several phase I and II clinical trials that employed an 18-base phosphorothioate ON (G3139) complementary to the first six codons of Bcl-2 mRNA have been reported [85–88]. The studies revealed that G3139 (Oblimersen) alone does not consistently produce a strong antitumor response. Therefore, subsequent trials investigated Oblimersen combined with chemotherapy. G3139, when combined with paclitaxel in a cytotoxic dose range has been well tolerated by patients with chemorefractory small-cell lung cancer [89]. Furthermore, it can be safely administrated with fludarabine, cytarabine, and granulocyte colony stimulating factor salvage chemotherapy in patients with refractory or relapsed leukemia [90]. In spite of the promising early results, Oblimersen has not achieved the desired outcome in several trials. The FDA's Oncology Drug Advisory Committee advised against approving it for metastatic melanoma due to the lack of effectiveness, as measured by response rate and progression-free survival in relation to its toxicity (low-grade fever, usually resolving within 1–2 days, nausea but no vomiting, and thrombocytopenia). In phase III trials Oblimersen failed to reach the primary endpoint in the

treatment of multiple myeloma. However, it did meet the primary endpoint for the treatment of chronic lymphocytic leukemia, though it failed to meet the secondary endpoints of time-to-progression and overall survival. In order to understand why Oblimersen failed to achieve the desired result, we need to examine its pharmacodynamics in detail to see if it got into cells and hit the target. This information will help us decide whether Bcl-2 is a good target or not. Only by understanding the pharmacodynamics in detail can we hope to achieve the desired outcome in this case and others like it.

5.2 c-myb

The c-myb protooncogene is a normal homolog of the avian myeloblastosis viral oncogene (v-myb). Human c-myb encodes MYB, a protein that is responsible for regulation of cell cycle transition and cellular maturation, primarily in hematopoietic cells. AS-ONs targeting c-myb have been shown to be successful in decreasing mRNA and protein levels of MYB, as well as inhibit proliferation of acute myeloid leukemia, chronic lymphocytic leukemia, and T-cell leukemia cells in culture by inducing apoptosis [91]. In a phase I clinical pilot study, an AS-ON targeting c-myb (LR3001) has been used to purge marrow autografts administrated to allograft-ineligible chronic myelogenous leukemia patients. CD34+ marrow cells were purged by exposure to LR3001 for either 24 or 72 h. Patients received busulfan and cyclophosphamide chemotherapy, followed by reinfusion of previously cryopreserved and purged marrow cells. Post-purging, the c-myb mRNA levels declined substantially in about 50% of patients. Analysis of BCR-ABL (breakpoint cluster region-Abelson murine leukemia viral oncogene homolog) expression in a surrogate stem-cell assay indicated that purging has been accomplished at a primitive cell level in more than 50% of patients. Cytogenetic analysis was performed at day 100 on patients who engrafted without the need for an administration of unmanipulated "backup" marrow ($n = 14$), and six out of 14 patients demonstrated a major cytogenetic response. However, conclusions regarding the clinical efficacy of ODN marrow purging cannot be drawn from this small pilot study [92].

5.3 Protein Kinase C-α

Protein kinase C-α (PKC-α) is a cytoplasmic serine/threonine kinase that belongs to the family of isoenzymes involved in signal transduction in response to growth factors, hormones, and neurotransmitters. It regulates cell proliferation and differentiation. Overexpression of this kinase causes uncontrolled proliferation of cancer cells in several tumors. The antitumor activity of antisense inhibitors of PKC-α has been illustrated in cell culture studies [26, 93] and in animal models [94]. A number

of clinical trials employing AS-ONs designed to target PKC-α have been carried out. Results from clinical studies reported by Nemunaitis et al. [93] and Yuen et al. [95] revealed that the antisense therapy is well tolerated, but the antitumor effect is poor. Subsequent data from phase II studies in patients with colorectal [96] and prostate [97] cancer confirmed the results – no significant antitumor activity was observed. Anti-PKC-α ODNs have also been studied in combination with 5-fluorouracil and leucovorin in patients with advanced cancer [98]. This combination therapy has shown antitumor activity, but also some toxicity including alopecia, fatigue, mucositis, diarrhea, anorexia, nausea, and tumor pain. In addition, a phase III study comparing LY900003 (Affinitac) plus gemcitabine and cisplatin versus gemcitabine and cisplatin in patients with advanced, previously untreated non-small cell lung cancer has been suspended due to lack of efficacy. Before antisense therapy to PKC-α succeeds, the pharmacodynamics of LY900003 needs to be thoroughly investigated to ensure that it is hitting its target.

5.4 H-Ras Pathway

Another attractive target for antisense therapy is H-Ras, a regulator of several interconnected signaling pathways. Constitutive activation of this gene promotes uncontrolled proliferation and malignant transformation in many human tumors. Several clinical trials have used AS-ONs directed against H-Ras (ISIS 2503). Results from a phase I study in patients with advanced carcinoma presented by Cunningham et al. [99] showed that ISIS 2503 gives only a partial response in some individuals and is associated with mild toxicity. However, stabilization of disease has been reported in several patients. ISIS 2503 tested in combination with gemcitabine has been well tolerated, and a partial response in patients with metastatic breast cancer has been documented [100]. The results of a more recent trial using ISIS 2503 in combination with gemcitabine to treat patients with pancreatic adenocarcinoma are promising [101]. A median overall survival of 6.6 months and a response rate of 10% were achieved. However, further work needs to clarify tumor characteristics that may help better predict response to the therapy. Downstream effectors of the RAS signal transduction pathway, like c-Raf, have also been investigated as potential targets for antisense therapy. Based on the encouraging results of in vitro experiments [102] and in vivo tumor xenograft mouse models [103], several clinical trials have been conducted. Although no major tumor response has been reported, some patients have shown a stabilization of their disease [104–106]. (With kind permission from Springer Science + Business Media [41, 42]).

Nucleic acid-based therapeutics are is still in their development phase and to achieve better results scientists are working on developing better chemistries to enhance the stability and potency and high efficiency of these molecules (Fig. 3) [19]. Apart from a perfect chemistry, delivery vehicles for these magic molecules are equally important for the success of this technology. A few of the

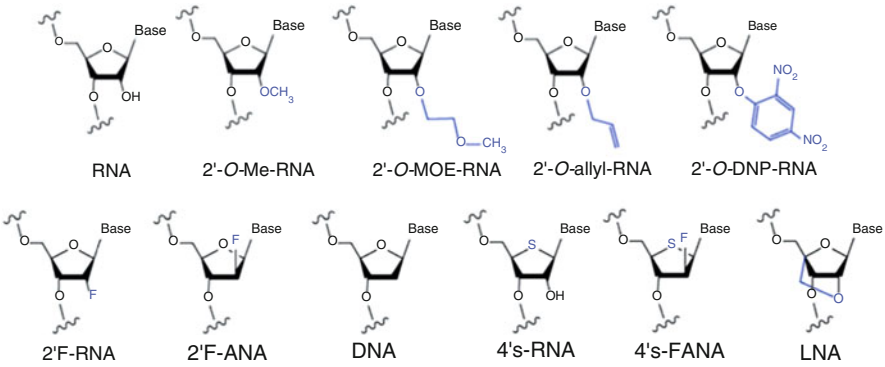

Fig. 3 Various modified sugar units. *First row*: 2′-*O*-alkyl (2′-*O*-Me, 2′-*O*-MOE, 2′-*O*-allyl) and 2′-*O*-aryl (2′-*O*-DNP) modifications. *Second row*: 2′-modifications (2′F-RNA, 2′F-ANA, DNA), 4′-modifications (40S-RNA, 40S-FANA), and locked nucleic acid (*LNA*), a conformationally constrained modification [19]. Image reprinted with permission from Elsevier

methodologies include the use of RGD-PEG-albumin conjugates that may help the delivery of ONs to certain cells [107]. Further, a protein transduction domain with a dsRNA binding domain fusion protein is also being used to delivery siRNAs [108]. There are reports on the use of aptamers for the delivery of siRNAs [109] and others describing the advances on gene transfer vectors [110]. Most recently, an efficient delivery approach was reported in humanized mice using CD4 aptamer-siRNA chimeras for inhibition of HIV transmission [111]. This strategy specifically suppressed gene expression in CD4+ T cells and macrophages in vitro, in polarized cervicovaginal tissue explants, and in the female genital tract of humanized mice without activating any lymphocytes or stimulating innate immunity. Further, a very recent report achieved gene silencing in a synergistic manner and obtained an efficient siRNA delivery by using a combination of ionizable lipid-like materials. Their results suggested that such a strategy is much better than using a single cationic or ionizable material for delivery [112]. All these approaches are encouraging and demonstrate the ability to translate to the next level of development of nucleic acid-based therapeutics.

6 Clinical Trials on Nucleic Acid Therapeutics

Isis, in collaboration with Genzyme, is developing a cholesterol-lowering drug, mipomersen, which is a second generation 2′-*O*-(2-methoxy) ethyl-modified ribose ON. Unlike conventional therapies, mipomersen acts directly on apolipoprotein B100, instead of inhibiting HMG-CoA reductase (as does Lipitor). Recent data showed that the drug is able to dramatically reduce low density lipoprotein (LDL) levels but also caused an unexpected increase in liver enzyme levels above the

normal limit in a certain percentage of patients [113, 114]. Overall, the data from two mipomersen phase 3 trials show that it meets primary endpoint, with 36% LDL-chloesterol reduction with patients suffering from severe familial hypercholesterolemia. Another second generation drug that targets clusterin (from Isis in collaboration with OncogeneX and Teva Pharmaceuticals) and has completed phase 2 studies. This drug in combination with docetaxel is well tolerated in patients with advanced metastatic castrate-resistant prostate cancer (CRPC). The data from phase 2 trials with 82 patients showed an overall median survival of around 24 months as opposed to around 17 months survival of patients only treated with docetaxel [115]. A phase 3 trial has also been started with custirsen (AS-ON targeting clusterin) in patients with metastatic prostate cancer [116]. Another phase 2 study is a trial from OncogeneX for CRPC patients using the drug code named OGX-427 [117]. Further, ELI Lilly recently finished a phase 2 study with OncogeneX for the development of LY2181308. This drug targets the protein survivin [118]. Some molecules are also in phase 2 trials for metabolic and inflammatory disorders. Two other ISIS drugs for diabetes worth indicating are ISIS-113715 [119] that targets PTP-1B, and ISIS-SGLT2$_{RX}$ that targets sodium glucose co-transporter type 2 and is their first drug with a target in kidney. These drugs are in phase 2 and phase 1 studies, respectively. Alicaforsen (with Atlantic Healthcare), ATL/TV1102 (with Teva and Antisense Pharma) and AIR645 (with Altair) are in phase 2 studies for ulcerative colitis, multiple sclerosis, and asthma, respectively. Another second generation AS-ODN, ISIS-FXIRx, has proceeded to the next level of clinical development for the treatment of thromboembolic disease. This drug showed a significant improvement in the anticoagulant and antithrombotic activity in preclinical trials.

Other AS-ON molecules in phase 3 studies include a 25-mer ON (Aganirsen) from Gene Signal that targets insulin receptor substrate-1, and Trabedersen (AP 12009) from Antisense Pharma that targets transforming growth factor-β (TGF-β) in patients with recurrent or refractory anaplastic astrocytoma [120]. In addition, Genta's Genasense, a modified 18-mer DNA, targets Bcl-2 mRNA and recently completed a study encompassing a double-blind, phase 3 trial of dacarbazine with or without Genasense (Oblimersen sodium) injection in patients with advanced melanoma [121]. However, this drug did not show any significant increase in overall survival compared with patients treated with chemotherapy alone[8].

Other approaches in the field include a new way to create a hostile environment for the hepatitis C virus (HCV) in the host liver cell instead of directly interfering with its cellular machinery [122]. This is achieved by using a locked nucleic acid (LNA) modified ON that targets miRNA-122, which is considered essential for HCV propagation in liver cells. Miravirsen (Santaris Pharma code SPC3649) is the first miRNA-targeting drug to enter phase 2a studies in the USA [123].

Further, ENZ-2968 and ENZ-3042 from Enzon are drugs for the treatment of solid tumors and lymphomas. These 16-mer chimeras with a phosphorothiolated segment of ONs in the middle flanked by seven LNA nucleotides on each side are inhibitors of hypoxia-inducible factor 1 alpha (HIF-1α) and survivin, respectively. According to the last published reports, ENZ-2968 is still in phase I/II studies.

Regulus Therapeutics, which has reported that longer anti-miRNAs are more potent than shorter ones targeting only the seed region, is also targeting miRNA-122 for HCV therapy. Regulus is also involved in oncology, fibrosis, immuno-inflammatory and metabolic disease anti-miRNA-based drug development programs in partnership with GSK and Sanofi Aventis.

Companies like Alnylam and Dicerna that specialize in siRNA have some molecules in initial clinical studies. Alnylam's lead compound ALN-RSV01 is being developed for the treatment of respiratory syncytial virus infection [124]. Based on the encouraging phase II results (in lung transplant patients) in collaboration with Cubist, the company has initiated a phase IIb multicenter, global, randomized, double-blind, placebo-controlled study [125]. Another major phase 1 study for liver cancer with molecule ALN-VSP that targets kinesin spindle protein and vascular endothelial growth factor shows that the drug is well tolerated and has controlled pharmacokinetic data, and is currently undergoing phase 1 trials [126]. Moreover, other companies like Dicerna, which are still trying to optimize the molecular chemistries of siRNA, are yet to come into preclinical trials. However, they use a novel enhanced substrate technology and claim that 27-mers are more potent and efficient substrates for Dicer than the conventional 21-mers. They also report that these long substrates have a long duration of action and thus can have a reduced frequency of dosage.

7 Conclusions

Gene silencing strategies have been evolving very rapidly over the past few years. The ability to block gene function with nucleic acids is now an accepted and powerful research tool, and these strategies have a significant therapeutic potential in the years to come. This field is still struggling for acceptance in the clinic because technical problems relating to targeting, stability, cellular uptake, and delivery are still unresolved. The studies described here give us significant insights on the importance of structure and the accessibility of target sites. The rules governing the relationship between RNA structure and effectiveness of siRNA therapy remain unknown and there is a need for a refinement of these techniques to efficiently identify the target sites and thus facilitate the process of development of AS-ODNs and siRNA-based therapeutics. The use of new chemistries for the development of these molecules can further improve stability and toxicity. Lastly, and most importantly, an array of efficient delivery vehicles need to be optimized to deliver these molecules, especially siRNAs, right to the target site. However overwhelming these issues may seem, there are a number of scientists who are looking at various aspects of these problems at the cross-platform level and we hope that they will be able to find an efficient and effective way to optimize efficiency and delivery of these molecules and make them accepted drugs in the clinic.

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