Peptide Conjugates of Oligonucleotides: Synthesis and Applications

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1. Introduction

Ever since the observation that synthetic oligonucleotides and their modified analogues can be used successfully to modulate the functions of specific genes,[1,2] the research on oligonucleotide based therapeutics has been intensified. It is believed that therapeutics based on oligonucleotides will become a reality in the future. The chemist's role in oligonucleotide science has become much more demanding. Oligonucleotides with a wide variety of modifications at different parts of the sequences have been designed and synthesized by chemists.[3−5] Their biological significance in inhibiting the gene functions by acting either as antisene, antigene, or aptamers has been evaluated by biologists. The siRNAs are recent additions to these series of oligonucleotide analogues to inhibit the gene functions, in a process known as RNA interference (RNAi).[6] Thus, a number of research groups, with chemists and biologists working together, are actively involved in expanding the subject further. Now, with the human genetic map in hand, after completing the "Human Genome project", they have started correlating the regions of the genes to their functions (functional genomics).[7]

Irrespective of all these magnificent developments, millions of people continue to suffer from one or another form of gene related diseases, worldwide. Especially, different types of carcinomas have taken a heavy toll of human life even in developed countries. Similarly, gene related defects at birth or at a latter stage also pose a significant threat to human life. Many of these diseases can only be contained by present day chemotherapeutics but cannot be cured. Oligonucleotide based therapeutic agents are strongly be-

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N. Venkatesan was born in India and received his undergraduate and postgraduate degrees in chemistry from the University of Madras, Chennai. He carried out his research work under the supervision of Prof. H. M. Chawla, Department of Chemistry, Indian Institute of Technology, Delhi, and subsequently received his Ph.D. degree in 2000. He started his postdoctoral research career as a Project Associate in the same laboratory and stayed there for about six months. During this period he was involved in the preparation of and studies on supramolecular aspects of calixarene based molecular receptors. Later, he worked as a postdoctoral research scientist with Prof. B. H. Kim's research group in POSTECH, Korea, in the same year, and he stayed there until December 2002. During this time he was involved in preparation of modified nucleoside analogues to be used as liposomes and hydrogelators. After a brief period of time, he joined the same research group again in June 2004 and continues to work on the research team now. He aspires to be a versatile researcher with wide interests. His present postdoctoral research work centers on the preparation of modified nucleoside and oligonucleotide analogues.

B. H. Kim was born in Busan, Korea. He got his undergraduate education at Seoul National University and obtained his M. S. degree from the Korea Advanced Institute of Science and Technology (KAIST) in 1979. Then he joined the Korea Research Institute of Chemical Technology as a researcher and stayed there until 1983. From 1980 to 1981, he had an opportunity to study at the Tokyo Institute of Technology (TIT) as a UNESCO fellow. He worked at the laboratories of Professor Isao Kuwajima and had valuable experiences with many group members. In the summer of 1983, he went to the U.S.A. for further study and was enrolled as a graduate student at the University of Pittsburgh. He earned his Ph.D. under the guidance of Professor Dennis P. Curran in 1987 and worked as a postdoctoral associate with Professor K. C. Nicolaou at the University of Pennsylvania from 1987 to 1988. After a one year stint in the K. C. N. group, he returned to his home country to become an Assistant Professor of Pohang University of Science and Technology (POSTECH). He was promoted to Associate Professor at POSTECH in 1993 and is now Professor and Head of the Department of Chemistry and Director of the BK School of Molecular Sciences. He was a visiting scholar at the University of Tokyo in 1995 and enjoyed his sabbatical leave in the laboratories of Professor Julius Rebek, Jr., at the Scripps Research Institute from 1996 to 1997. In 1999, he received the Jang Sehee Organic Chemistry Award given by the Korean Chemical Society (Organic Chemistry Division) and was selected as a 2000 Lectureship Awardee by The Society of Synthetic Organic Chemistry, Japan. He also received the Sigma-Aldrich Chemist Award (Korean Chemical Society) last year.

lieved to be a cure for these diseases. Unfortunately, even after almost two decades of research on oligonucleotides, we still lack an effective oligonucleotide based therapeutic against any of these dreaded diseases. The reasons for the failure are manifold. Important among them are the following: (a) poor cellular uptake of naked oligonucleotides, (b) lack of stability in intracellular fluid, (c) lack of target specificity of the oligonucleotides, and (d) low binding affinity to the target. There have been concerted efforts, using different tactics,[8,9] to surpass all, or at least most, of these barriers, to realize oligonucleotide-based therapeutics.

The biological significance[10] of peptides and proteins is very well studied and is better understood compared to that of oligonucleotides.[11] In fact, many peptide and protein based vaccines are in use. They are either isolated from natural sources or synthesized in the laboratory. In addition, peptides play a vital role in many biological processes. They function in various forms, such as hormones, antibodies, receptors, etc. Most important of all, peptides have better membrane permeability than the oligonucleotides. Also, peptides play a vital role in enhancing the stability of the cargo (by protecting it from digestion by intracellular enzymes) and in target specific delivery. Because of these features, peptides are used as carriers to internalize virtually any functionality. The size of the cargo may vary from small drug molecule to big macromolecules, which are otherwise membrane impermeable. Thus, different types of peptides capable of transporting such molecules into cells have been used either in the form of complexes[12] or in the form of covalent conjugates[8,13−15] to enhance their cellular uptake. Natural peptides derived from viral, bacterial, insect, and mammalian proteins as well as many synthetic peptides have been used for this purpose.

1.1. Peptide Oligonucleotide Conjugates

Significantly, the oligonucleotides are shown to be taken up by cells when they are administrated as noncovalent complexes or covalent conjugates of suitable peptides. There are a number of review articles on the use of different kinds of peptides, such as CPPs,[16−19] arginine rich MTS peptides,[20] Tat,[21] and some histidine-rich peptides,[22] to enhance the cellular permeability of oligonucleotides in cultured cells. These two distinct classes of biologically important biopolymers (peptides and oligonucleotides) are linked by covalent bonds in many possible ways, resulting in a new class of compounds known as peptide−oligonucleotide conjugates (POCs). POCs are also called peptide−oligonucleotides (POs) or oligonucleotide−peptide conjugates (OPCs). Nucleopeptides are well-known examples of naturally occurring peptide oligonucleotide conjugates. A detailed study on various aspects of these POCs assumes significance owing to their emerging applications in diverse fields. There are multitudes of reviews focusing on either synthesis[23−25] or cellular uptake[26,27] of POCs. Use of POCs as fluorescent probes, PCR primers, molecular tags,[28] etc. receives sporadic mention in some of these articles.

In most of the POCs studied, it is only the oligonucleotide fragment that gains an advantage upon conjugation to suitable peptide fragments. However, in the recent past, a couple of reports on POCs have suggested that the peptide fragments also gain some advantages upon conjugation to oligonucle-

otides. One such aspect is the ability to fine-tune the oligonucleotide cleavage activity of a certain peptide (e.g. artificial nuclease) by conjugating it to target specific complementary oligonucleotide sequences. Similarly, the molecular basis of protein synthesis, an important function conserved from prebiotic forms of life, can be understood well by POCs or aminoacyl-[29] or peptidyl-tRNA[30] models.

1.2. Types of Peptide Oligonucleotide Conjugates

A peptide can be conjugated either at the base-, 3′-, or 5′-position of the sugar unit or at the backbone of the oligonucleotide. Similarly, the point of conjugation in a peptide can be either the C- or N-terminus or the side chain. Different types of POCs known so far are depicted in Chart 1. They have been classified based on the points of linkages between the oligonucleotide and peptide fragments. We provide a comprehensive account on synthesis as well as applications of these fascinating bioconjugates.

Chart 1. Types of Peptide Oligonucleotide Conjugates

C- terminus or N- terminus or side chain of peptide attached to nucleobase of oligonucleotide directly or through linker

(C - nbO)
(N - nbO)
(scP - nbO)

C- terminus or N- terminus or side chain of peptide attached to oligonucleotide-sugar directly or through linker

(C - 5′)	(N - 5′)	(scP - 5′)
(C - 3′)	(N - 3′)	(scP - 3′)
(C - 2′)[a]	(N - 2′)[a]	(scP - 2′)[a]

C- terminus or N- terminus or side chain of peptide attached to backbone (phosphate) of oligonucleotide directly or through linker

(C - bbO)
(N - bbO)
(scP - bbO)

sc - side chain; nb - nucleobase; bb - backbone;
P - peptide; O - oligonucleotide
[a]possible in RNAs only

1.3. Modified Oligonucleotide Peptide Conjugates

Many kinds of modifications have been invoked to natural oligonucleotides to minimize the undesired properties and to maximize the desired ones.[3−5] Some of the modified analogues are shown in Figure 1. The oligonucleotide phosphorothioates (PS-DNA), 2′-*O*-methylribonucleotides (2′-OMe RNAs), and locked nucleic acids (LNAs) are shown to be the most successful among them. The modified analogues have been used in antisense techniques, and the subject is constantly progressing.[31] Enhancement of target (RNA or DNA) binding affinity, increasing stability, and reducing the toxicity are some of the aspects considered during such modifications. The LNAs are composed of conformationally locked monomers of 2′-*O*-4′-*C*-methylene-β-D-ribofuranosyl units joined by the same phosphodiester linkages as in natural nucleic acids, as shown in Figure 1.

The LNAs are shown to display very high affinity for Watson−Crick base pairing with DNA or RNA. LNAs have shown increased nuclease stability compared to natural nucleic acids, they are not easily degraded in serum or cell extracts, and they been used in *in vivo* experiments (in rats) without any detectable toxic reactions.[32]

Among the aforementioned modified oligonucleotide analogues, the peptide nucleic acids (PNAs) are more frequently used. In PNAs, the phosphodiester backbone is replaced by a pseudopeptide bond composed of *N*-(2-aminoethyl)glycine units. Unlike RNAs or DNAs, the PNAs have uncharged or neutral peptide backbones, and so they offer several advantages over the natural nucleic acids. Due to the absence of a negatively charged backbone, the strand invasion of a PNA on the DNA duplex forms a stable PNA−DNA hybrid with high mismatch discrimination.[33,34] The PNA−DNA hybrids are shown to be more stable than their homologous DNA−DNA hybrids.[35] Moreover, due to their unusual structure, PNAs are relatively more stable against both nucleases and proteases present in the human serum and cellular extracts.[36] Therefore, a number of antisense and antigene PNAs have been synthesized and studied.[37] But the potential application of PNAs is also hampered by their poor intracellular delivery. This problem has been addressed by using different delivery systems for antisense PNAs,[38] which include conjugation of PNAs to lipophilic moieties, peptides, and cell-specific receptor ligands. The biological activity of PNAs can also benefit from conjugation to DNA interactive compounds such as intercalators and alkylators.[37]

Phosphorodiamidate morpholino oligomers (PMOs) are a class of uncharged single-stranded DNA analogues modified such that each subunit includes a phosphorodiamidate linkage and morpholine ring as shown in Figure 1. PMOs can bind complementary sequences of RNA and inhibit gene expression by preventing translation or by interfering with pre-mRNA splicing.[39] Recently, PMO antisense agents have been reported to effectively interfere with the replication of several positive-strand RNA viruses in cell culture.[40] The sequence specificity, biostability, and low toxicity of PMOs make them good antisense agents to study gene function; however, their limited cell permeability limits their potential use. At present,

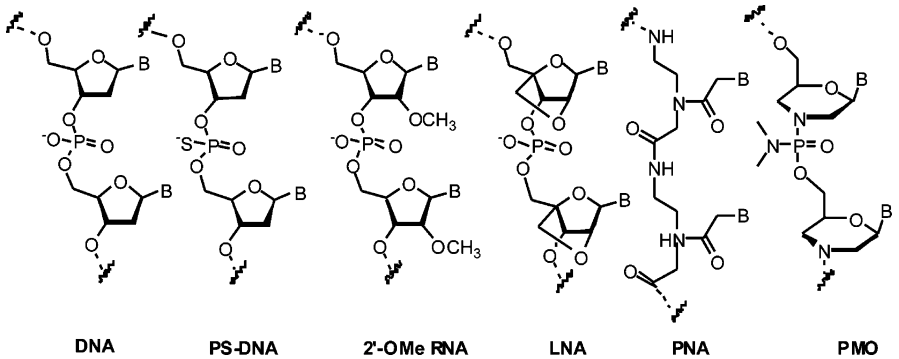


Figure 1. Structure of modified oligonucleotide analogues.

in most cases, the PMOs are delivered by mechanical methods. The ability of the HIV Tat peptide,[41] arginine-rich peptides,[42,43] and other cationic peptides to deliver PMOs into cultured cells has been investigated.

Oligonucleotides containing more than one kind of modified nucleoside analogue, called mixmers, have also been synthesized and used in antisense therapy. Different LNA/DNA sequences, called LNA/DNA mixmers, have attracted considerable attention as a possible antisense molecule for down-regulation of gene expression.[32,44] In the case of LNA/DNA mixmers, the melting temperature is reported to increase by 3-5 °C per substituted base for hybridization to DNA, giving the highest increase per modified base in mixed oligomers with every third base substituted.[45,46] So, a LNA oligomer as short as nine bases might have melting temperatures up to 80 °C and still be sensitive to mismatches, as in unmodified nucleic acids. Similarly, duplex LNA/DNA hybrids has been used as a decoy for the transcription factor NFkB.[47] Similarly, a 12-mixmer composed of 2′-OMe RNA and LNA residues complementary to HIV-1 TAR is reported to block Tat-dependent trans-activation in a HeLa cell assay when delivered by cationic lipids.[48]

1.4. Types of Chemical Linkages Used in Conjugation

A variety of chemical linkages have been used to link the peptide and oligonucleotide fragments, as can be seen in Table 1. A detailed description on the synthesis and utility

Table 1. Linkages Commonly Used in Conjugation of Oligonucleotides with Peptides

linkage	ref
N-acylphosphoramidate	99
N-alkylphosphoramidate	248
amide	101−104, 130, 132
disulfide	48, 50, 51, 113, 134
thioether	111−115,[a] 132
thiazolidine	119−122, 124, 125
oxime	106−108, 119−122, 124, 125, 128
urido	109, 110
hydrazone	122, 125, 215
thioester	116−118, 215

[a] Maleimido ether.

of POCs with these linkages is given respectively in sections 2 and 4 (*vide infra*). At least at present, we believe that the choice of a chemical linkage is mainly determined based on its easy synthetic accessibility. In a few biological experiments, the nature of the linkage between the two fragments is believed to play a role; however, this concept is refuted by other biologists. The disulfide bond of peptide conjugates of the type CPP−S−S-cargo is reported to be reducible in intracellular milieus.[49] This easy reducibility of the disulfide bond is reported to be a desirable aspect in enhancing the cellular uptake and antiviral activity of certain PNA−peptide conjugates.[50] But, in certain other cases, the stably linked conjugates are shown to be equal or even superior to their disulfide linked counterparts.[51]

A study on antisense activities of PMO−peptide conjugates containing chemically variable linkers concludes that the linker length and not the nature of the linkage (type of bond) plays a role in enhancing their antisense effects.[43] It was also established that the target RNA cleavage efficiencies of PNA−peptide conjugates with two different linkers are strongly dependent on the type of spacer connecting the PNA and the peptide.[52] Thus, the exact role or importance of the nature of chemical linkages is still to be explored. Similarly, the importance of the sequence specificity of the linkage i.e., C-terminal conjugation or N-terminal conjugation of a given peptide to 3′- or 5′-termini of the oligonucleotides, is also not studied properly, barring a few reports, for example in the works of Turner et al.[48]

In this review, various aspects related to chemistry and the role of chemistry in biological applications of these compounds are described. We have started with a discussion on different synthetic strategies adopted to prepare the conjugates, in section 2, and then we have described different methods of purification and characterization of the conjugate, in section 3. In section 2, we have also discussed the protecting groups, deprotection conditions, and other reaction conditions used so as to obtain the POCs with ease and in better yield. In section 4, we have briefly discussed the recent reports on the usage of peptide conjugation to improve the therapeutic value of oligonucleotides and their modified analogues such as oligonucleotide phosphorothioates, mixmers, LNAs, PNAs, PMOs, etc., quoting recent publications on the subject. For better understanding, the applications are subdivided based on the nature of applications (antisense, antigene, vaccine, and gene silencing) rather than the nature of modifications (PNA, PMO, etc.) or the type of conjugations (as described in Chart 1). An update on mechanistic studies of internalization of POCs has also been described. Nontherapeutic applications of POCs, such as in diagnostics, detection, isolation, and purifications, have also been discussed. Overall, we believe this article supports the idea that peptide oligonucleotide conjugation is an important subject to study.

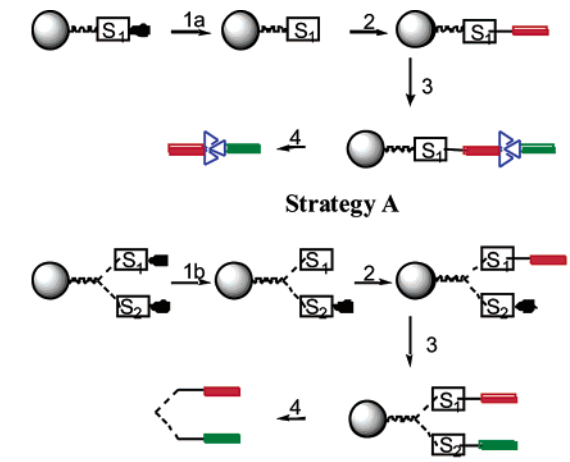
2. Synthesis

Two different strategies have been adopted for the synthesis of POCs:[53−55] they are on-line solid-phase synthesis (divergent method) and fragment conjugation (convergent) methods. In solid-phase methods, the peptide and oligonucleotide fragments are assembled, sequentially on the same solid support, until the final step. In the fragment conjugation, the peptide and oligonucleotide fragments synthesized individually are linked postsynthetically.

2.1. Solid-Phase Stepwise Synthesis (Divergent Methods)

The synthetic strategies used for sequential solid-phase synthesis of POCs are summarized in Chart 2. The POC is synthesized first by assembling oligomer A followed by assembly of oligomer B. Oligomer A contains a masked but reactive functional group at the site of conjugation. The point or site of conjugation in oligonucleotides and peptides can be the termini or side chain, as described in section 1.2 (see Chart 1). The sequence of assembly can be either "peptide-first−oligo-next" or "oligo-first−peptide-next". In predominant cases, the peptides are assembled first by the Fmoc or Boc method (i.e. oligomer A in Chart 2 is the peptide), while the oligonucleotides are assembled next (i.e. oligomer B in Chart 2 is the oligonucleotide) using the phosphoramidite method. One of the major blocks in using the solid-phase sequential method is the mutual incompatibilities of the reaction conditions used for the syntheses of oligomer A and oligomer B. Especially in the "oligo-first−peptide-next"

Chart 2. Common Synthetic Strategies Used for the Stepwise Solid-Phase Synthesis of Peptide Oligonucleotide Conjugates



S_1 - site 1; S_2 - site 2;
Pf - protected reactive functionality

= protecting group = solid support
= oligomer A (peptide in general)
= oligomer B (oligonucleotide in general)

1a. Deprotection of S_1P; 1b. Selective deprotection of S_1P.
2. Step-wise construction of oligomer A
3. Deprotection of S_2P followed by step-wise construction of oligomer B
4. Cleavage from solid support

strategy, the acid-labile oligonucleotides have to be exposed to the relatively harsh conditions used for peptide synthesis. During this time, the oligonucleotide chains are prone to undergo cleavage and depurination. On the other hand, in the "peptide-first−oligo-next" strategy, the peptides assembled first remain stable during oligonucleotide synthesis. However, it must be noted that peptides may not always be stable under ammonia deprotection conditions. In addition, the protecting groups, activating and deblocking agents, used during synthesis of oligomer A are not more useful for assembly of oligomer B. Hence, proper choice of protecting groups, and other reaction conditions, is quite crucial for better yield and easy isolation of the products.

In the recent past, significant advancements have been made in chemical synthesis of POCs. Discovery of improved polymer supports, newer sets of protecting groups for exocyclic amino groups of nucleobases, and better coupling reagents are important among them. These improvements, along with the introduction of high throughput synthesizers, have made a notable contribution to the growth of peptide oligonucleotide conjugation chemistry. Methods available for preparation of a number of oligonucleotide conjugates with biological and abiological ligands have been reviewed.[56−59]

2.1.1. Solid-Phase-Immobilized Linkers Used in Sequential Synthesis of POCs

As depicted in Chart 2, the first step in preparation of various peptide oligonucleotide conjugates involves modification of solid supports with suitable linkers. A number of

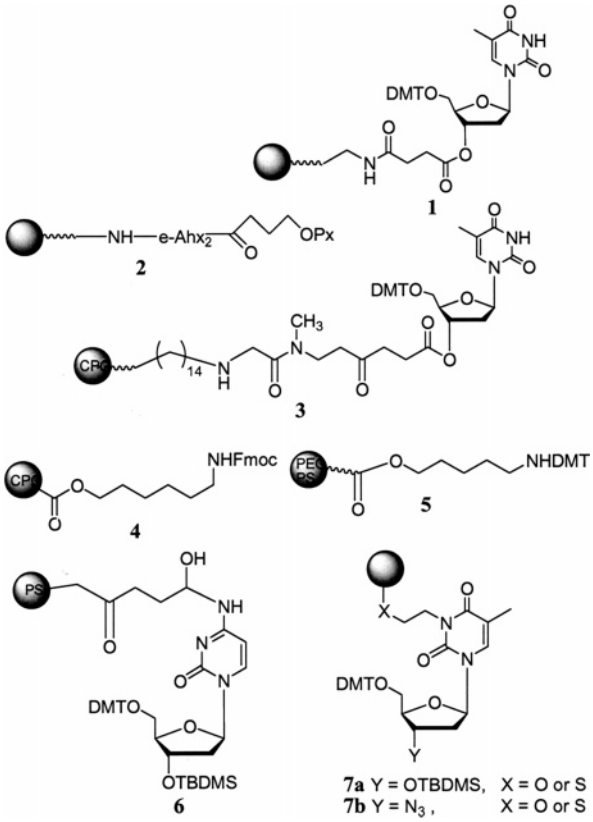


Figure 2. Monofunctinalized linkers used in the solid-phase synthesis of conjugates.

monofunctionalized as well as bifunctionalized linkers were immobilized over solid supports through suitable spacers. Some of these linkers are listed in Figures 2 and 3.

2.1.1.1. Monofunctionalized Linkers. Grandas' research group has long been involved in the syntheses of various nucleopeptides.[60] In some of the natural nucleopeptides, the side chain hydroxyl group of the peptides containing serine, threonine, or tyrosine is linked to the 5′-terminus of the oligonucleotide by a phosphonate diester linkage, as in Chart 1 (scP→5′). They have used different solid supports to obtain nucleopeptides. For example, PS supports modified with a nucleotide building block (**1**, Figure 2) have also been used.[61] The oligonucleotide was assembled by phosphoramidite chemistry, and then the Boc-Ser[O−P(OCEN)N(^{i}Pr)$_2$]cHex was coupled by the phosphite triester method. Fmoc and cyanoethyl groups were used to protect the exocyclic cytosine amine and phosphate, respectively. Final deprotection was carried out by treatment with methanolic K_2CO_3. Deprotection followed by cleavage gave the model nucleopeptide Boc-Ser(pTCT)-NHcHex. Using this strategy, the authors have prepared adenovirus-2 nucleoprotein analogues such as the tripeptidylhexanucleotide Ac-Ser(pCATCAT)-Gly-Asp-NH_2.[61]

Haralambidis and co-workers have used the modified CPG supports such as **2** (Figure 2) to synthesize POCs in the sequential solid-phase method.[62] This monofunctionalized solid support is prepared first by attaching ϵ-Ahx followed by 4-OPx protected 1,4-hydroxybutyric acid. The acid-labile OPx is removed by treatments with 3% v/v DCA in DCM, prior to peptide assembly. The peptide is assembled by the Fmoc method. Then, the N-terminus of the peptide sequence is linked to 4-OPx protected 1,4-hydroxybutyric acid. The OPx group is deprotected and elongated with the ODNs by the standard phosphoramidite method through the 3′-terminus of the ODN to obtain (3′→N) type POCs. The purified

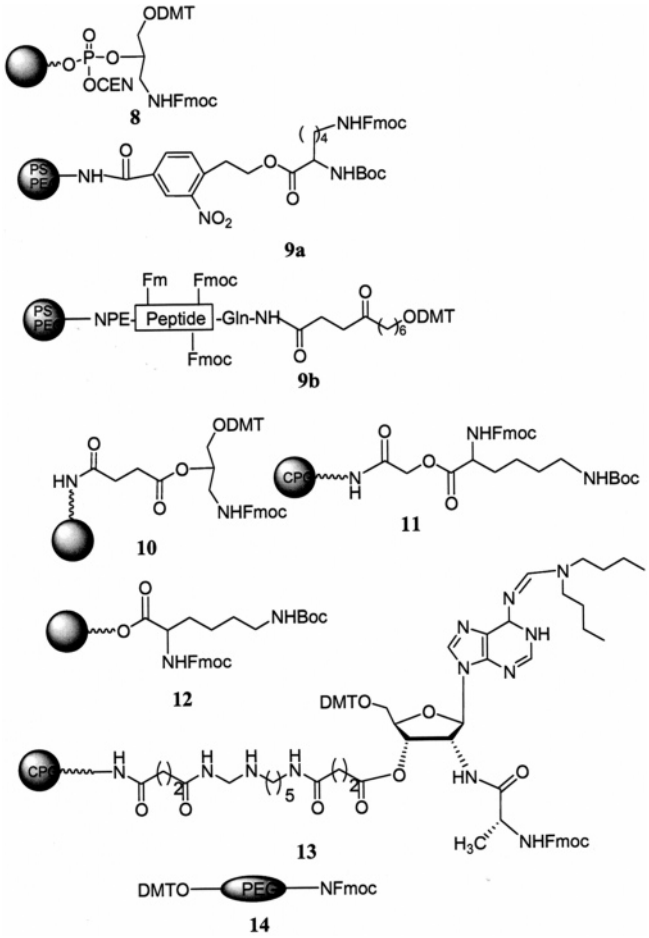


Figure 3. Difunctionalized linkers used in the solid-phase synthesis of conjugates.

conjugates were obtained in milligram scale. They were characterized extensively by several analytical techniques including ion spray mass spectrometry. The versatility of this method has been demonstrated by synthesizing a variety of peptide−ODN conjugates.

Bannwarth and co-workers have reported a general methodology for the solid-phase synthesis of directly linked chimeric DNA−peptide−DNA conjugates[63] wherein both the C- and N-termini of the peptides are conjugated to oligonucleotides. They have used a sarcosine modified CPG support (**3**, Figure 2) to obtain the conjugates. The oligonucleotide was first assembled using the usual phosphoramidite method. Then, the peptides were assembled using the Fmoc method, followed by oligonucleotide assembly on the N-terminus of the previous POC to yield the DNA−peptide−DNA hybrid. Stetsenko and Gait have used an ω-aminoalkylsuccinate/L-homoserine combination linker to synthesize a variety of (3′→N) oligonucleotide peptide conjugates.[64] The CPG as well as macrophorous PS and Argopore resin were used as solid supports.[65] The solid support is modified with sarcosine followed by an *N*-Fmoc protected aminohexylsuccinate to get a modified support of type **4** (Figure 2). The peptide is assembled first using Fmoc chemistry. After the peptide assembly, Fmoc-Hse(Trt)-OH is manually linked to the N-terminus of the peptide. This peptide-loaded resin is then subjected to oligonucleotide assembly by the normal phosphoramidite method. This so-called "homoserine-linker approach" has been successfully extended to synthesize a wide variety of POCs. A number of cell or nuclear penetrating peptides could be conjugated to oligonucleotides containing 2′-deoxynucleoside, 2′-*O*-methylribonucleoside phosphodiester, or gapmers containing 2′-deoxyphosphorothioates.[65] Using this route, even a fluorophore label could be attached to POCs.[66]

Thus, the solid support has been used as a universal support to synthesize 3′-conjugates of oligonucleotides. The solid support was prepared first by attaching a spacer and then an L-homoserine branching unit. The N^{α} position of the homoserine is protected by an Fmoc group, while the side chain hydroxyl is protected with a trityl group. The Fmoc is base-labile (cleaved by piperidine) while the trityl group is acid-labile (cleaved by TFA). The solid surface is modified first with sarcosine followed by attachment of aminohexyl succinate (as a spacer). This modified solid support could be prepared easily by peptide type reactions, and the support was stable enough to prepare a wide variety of POCs under different reaction conditions.[65]

A sarcosyl modified monofunctional PEG-PS support carrying a 5-aminopentyl linker (**5**, Figure 2) has recently been used for the synthesis of POCs.[67] The peptide was first assembled by Boc chemistry, and then the oligonucleotides were assembled using the phosphoramidite method. One of the oligonucleotides was synthesized by the (5′→3′) (reversed phosphoramidite) method. After complete assembly of the peptide, the N-terminus is linked to an active ester of *N*-DMT protected 6-hydroxybenzoic acid as linker. It is notable that, in the case of PEG-PS supports, the coupling yields are influenced by the presence of amino acids. This is believed to be due to the influence of amino acids in swelling of the PEG-PS support.

Piccialli and co-workers have also developed a nucleoside based polymeric support for synthesis of 3′- as well as 5′-peptide conjugates of oligonucleotides.[68,69] Initially, they designed and synthesized a cytidine based support (**6**, Figure 2) for preparation of 17-mer oligonucleotide 3′-peptide conjugates. The conjugates were obtained in good isolated yields. In a typical example, the 3′-terminus of an oligonucleotide sequence (antisense to HIV-1) was linked to the tripeptide, Gly-Gly-His, in good yield. Recently, they have also reported the synthesis of two thymidine based polymeric supports (**7a** and **7b**, Figure 2), wherein the thymidines were attached to a β-hydroxythioether functionalized resin through the thymine moiety, using a Mitsunobu reaction. Both the 5′- and 3′-termini of this solid support are available for further functionalization. After complete assembly of POCs, the nucleosidic material can be released from the solid support by classical ammonia treatment. Starting from these supports, the authors have devised a versatile synthetic strategy to obtain a variety of thymidine hybrids following well-established peptide and oligonucleotide chemistry. It is stated that this methodology can be easily exploited to link any other amido- or imino-functionalized heterocycle to a solid support.[69]

2.1.1.2. Bifunctionalized Linkers. In addition to the aforementioned monofunctionalized linkers, a number of research groups have also reported the successful use of bifunctionalized linkers. In the case of bifunctionalized linkers, the two sites are orthogonally protected. The most commonly used orthogonal protecting groups are DMT and Fmoc, respectively, for oligonucleotide and peptide assembly sites. Lysine is one of the most commonly used bifunctional linkers. In general, the C-terminus of lysine is linked to the solid surface while the N^{α}- and N^{ϵ}-termini are used for peptide and oligonucleotide assembly in a "peptide first−

oligo-next" way.

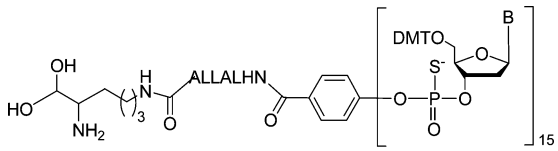
Brousseau and co-workers have used, for the first time, a Teflon support for the synthesis of POCs,[70] wherein the authors have synthesized (3′→N) conjugates of an oligonucleotide 17-mer with different peptides. The commercially available Teflon support was modified with a bifunctional linker (**8**, Figure 3). The peptide was assembled first on linker **8** by using Fmoc chemistry, and then the oligonucleotide was assembled using phosphoramidite chemistry. The DMT deprotection was carried out with ethylenediamine in absolute ethanol. Finally, the conjugates were cleaved from the solid support by treatment with a dilute solution of periodate.

Eritja and co-workers have used a PEG-PS support (NPE resin), modified with lysine as a bifunctional linker (**9a**, Figure 3).[71] The two different sites, namely, the α- and ϵ-amino groups of the lysine, were protected respectively by Boc and Fmoc groups. First, the peptide was assembled using the Boc protocol. The side chain amino groups were also protected by Fmoc. After complete assembly of the peptide sequences, the N-terminus of the peptide was modified with a spacer containing a DMT protected hydroxyl group (**9b**, Figure 3). Then, the oligonucleotide was assembled using the phosphoramidite method. Base-labile protecting groups were used for the amino acid side chains and nucleobases. It may be noted that use of CPG was not efficient, as it gave only a mixture of peptides with different chain lengths. Also, they have used a DCM solution of phosphoramidite and a solution of tetrazole in dry acetonitrile or THF to get a coupling efficiency of 90% or above.

Peptide conjugates of oligonucleotide phosphorothioates have been prepared using solid-phase-immobilized bifunctional linkers of type **10** (Figure 3).[72] The two different sites, respectively, for peptide and oligonucleotide syntheses are protected with Fmoc and DMT groups. A number of 15-mer oligonucleotide phosphorothioates containing the 3′-terminal peptides have been prepared using this support. The peptides were linked to the oligonucleotides through their C-termini.

Similarly, the same research group has also reported successful use of N^{α}-Fmoc, N^{ϵ}-Boc protected lysine containing solid supports of type **11** (Figure 3) for POC synthesis.[73] In **11**, the lysine is linked to the solid support by convenient base-labile ester linkages (through its C-terminus). In both methods, the peptide was assembled first on the Fmoc protected amino function, using standard Fmoc peptide chemistry. Commercially available N^{α}-Fmoc protected amino acids could be used directly. The N-terminus of the peptide is Boc deprotected and subsequently attached to the *O*-DMT protected hydroxyacetic acid linker. Then, the oligonucleotide phosphorothioates are assembled using a standard protocol.

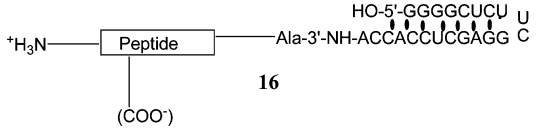
It is reported that the phosphate diester bond derived from the aliphatic hydroxyl function was unstable in concentrated aqueous ammonia. Such a phosphate diester bond connecting peptide and oligonucleotide fragments is broken up by β-elimination.[74] In an effort to alleviate this problem, Zhang and co-workers have used linkers containing no β-protons.[75,76] For example, they first used *p*-hydroxybenzoic acid as a linker connecting peptide and oligonucleotide fragments. The hydroxyl modified CPG surface was first linked to N^{ϵ}-Boc, N^{α}-Fmoc protected lysine by a base-labile ester link (**12**, Figure 3).[76] The peptide was synthesized first by the Boc strategy. However, the acidic deprotection procedure did not have any adverse effect on the ester link between the peptide and the solid phase. After complete assembly of the peptide sequences, the *p*-hydroxybenzoic acid was linked to the N-terminus of the peptide, to avoid cleavage of the phosphodiester bond by β-elimination. The resultant CPG was submitted to further synthesis of phosphorothiolated ODNs, to obtain POCs of type **15**.



15

The same group has also successfully used 2,2-dimethyl-3-hydroxypropionic acid as a linker to prepare a mimetic signal peptide conjugated to oligonucletide phosphorothioates.[75] The resulting POC was quite stable in the basic conditions.

Strazewski and co-workers have used PS 50% cross-linked with *p*-divinyl benzene as a solid phase to synthesize (N→3′) peptidyl-tRNA analogues of type **16**.[77] The solid phase is



16

modified with a long spacer followed by an adenoside based linker group, as shown in **13** (Figure 3). The bifunctionalized 3′-alanylamino-3′-deoxyadenosine is orthogonally protected with Fmoc and DMT groups. The Fmoc and phosphoramidite chemistries were used respectively for the assembly of peptide and oligonucleotide sequences. After completion of the stepwise oligopeptide and then oligoribonucleotide synthesis, the crude conjugates were deprotected and detached from the solid support using methylamine.[77] The peptide is linked through a stable amide bond to the tRNA at the 3′-terminus of a ribonucleotide.

A new liquid-phase procedure for the preparation of peptide−PEG−oligonucleotide conjugates has been reported[78] wherein oligonucleotide and peptide sequences were synthesized at the extremities of the same commercial high molecular weight PEG support (**14**, Figure 3). The terminal hydroxyl functions of this soluble support are protected with two orthogonal protecting groups: DMT and Fmoc. This modified PEG has been used both as support and as linker (or conjugating agent). It has been shown that it is possible to synthesize a chimeric oligonucleotide−PEG−peptide conjugate in a liquid-phase process using this support. Also, the synthesis by either "peptide-first−oligo-next" or "oligo-first−peptide-next" fashion could be carried out without any substantial difference in overall yield of the conjugates.[78]

2.1.2. Protection Methods

From Chart 2, it is evident that, up to the construction of oligomer A, the standard protocol used for the synthesis of peptides or oligonucleotides can be used without any difficulty. But, the construction of oligomer B becomes problematic. The nucleic acids, in particular the DNAs, are very acid-sensitive. But, the amino acid side chain protections commonly employed in peptide chemistry require strong acidic conditions for their deprotections. In such conditions, DNA undergoes depurination. In an effort to protect the nucleic acids from being exposed to these harsh conditions, almost all the POC syntheses reported so far have been

performed by the "peptide-first−oligo-next" strategy; that is, oligomer A, in most cases, is the peptide while oligomer B is the oligonucleotide.

The side chain protecting groups used for assembly of oligomer A (peptide in most cases) must be compatible with the reaction conditions used for the subsequent assembly of oligomer B (oligonucleotide). Much effort is being devoted toward this end by design and execution of different protection strategies. Some of them include using different sets of protecting groups, for the same functional group, during assembly of oligomer A and oligomer B, i.e., use of transient protecting groups. However, an ideal condition would be use of uniform protecting groups throughout the POC assembly.

In general, the main criteria that should be kept in mind while choosing the suitable side chain protecting groups are as follows: (a) it should allow efficient assembly of a peptide chain without interfering in the coupling reactions; (b) the removal of a protecting group should not require a hard acidic treatment (TFA etc.) but rather basic or mild acidic conditions (this is the reason base-labile protecting groups have been used in most cases); and finally, (c) it is preferable to use commercially available side chain protecting amino acid building blocks, especially in the automated synthesizers.

2.1.2.1. Protection of Side Chain Amino Acids. Some of the groups used for different amino acid side chains are listed in Table 2. Truffert et al. have reported a general

Table 2. Protecting Groups Used for Amino Acid Side Chains

amino acids[a]	protecting groups used	ref
cysteine (Cys)	Trt	79
	Acm	73
aspartic acid (Asp)	Fm	76, 82−84, 103
	Dmab ester	65
glutamic acid (Glu)	Allyl ester	77
	Bzl	73
histidine (His)	Tos	81, 84
	Dnp	84
	Trt	65, 73
	2,6-Dmbz	86
lysine (Lys)	Boc	79
	Dde	80
	Fmoc	82−84, 103
	Tfa	65, 73, 83−85, 103
methionine (Met)	As methionine sulfoxide	84
asparagine (Asn)	N^{α}-Fmoc-Orn(Mtt)-OH as precursor	67
arginine (Arg)	Fmoc	83, 84, 103
serine (Ser)	tBu,	73
	TBDMS	79
tryptophan (Trp)	formyl	28, 53, 103
	Boc	73
tyrosine (Tyr)	2-ClTrt[b]	65
	iBu	65
	tBu	73
orinithine (Orn)	Mtt	85

[a] The amino acids glycine, alanine, phenylalanine, leucine, isoleucine, and valine do not require side chain protection. [b] Used as a transient protecting group.

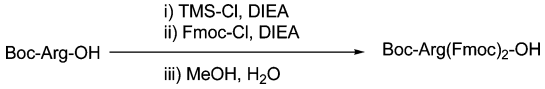
automated procedure for the synthesis of oligonucleotide 3′-peptide conjugates.[80] They obtained the most satisfactory results using commercially available, Fmoc-Lys(Dde)-OH, building block for introduction of lysine. The Dde side chain protecting group is reported to be well suited, as it is stable in 20% piperidine (for Fmoc deprotection) but can be cleaved under basic conditions with ethanolamine or hydrazine solutions. Thus, treatment of conjugates with ethanolamine in absolute ethanol (1:1) under argon for 30 h at 60 °C cleaved the conjugates from the solid support, and the Dde group was simultaneously deprotected. Here, concentrated ammonia could not be used, as it gave a mixture of products.

The same research group has also reported a procedure for introduction of histidine into POCs.[81] Here, they used a commercially available side chain tosyl protected, Boc-His-(Tos)-OH, building block. To minimize the racemization of His(Tos), they used only a catalytic amount of DMAP (0.1 equiv). The tosyl group was easily removed with NH_4OH or NaOH solution at low concentration without side reactions. They have used pentafluorophenyl ester (Boc-His(Tos)-OPfp) and triethylamine for effective coupling of this building block.

The base-labile Fmoc and Fm groups have also been used for the side chain protection of lysine and aspartic acid, respectively.[71,82] These protecting groups, along with the nucleobase protecting groups (^{i}Bu for G; Bzl for A and C), could be removed by treatment with concentrated aqueous ammonia in dioxane. A number of oligonucleotide 3′-peptide conjugates were synthesized by using these protection strategies. Also, the C-terminal amino acids of these peptides were shown to be stable against racemization under these basic deprotection conditions.[82] The authors have also studied the stability of amino acid side chain protecting groups during oligonucleotide synthesis. Solid supports containing the peptide sequences were subjected to oligonucleotide synthetic conditions. Thus, several solid-phase immobilized dipeptides of the type Boc-[Gln, Asp(Fm), or Lys(Fmoc)]-Phe were prepared and exposed to reagents used for the capping, oxidation, and coupling of oligonucleotide synthesis. None of these reagents yield any unwanted side products, indicating that the peptides containing these amino acid blocks are stable during oligonucleotide synthesis.

Grandas and co-workers have also successfully conjugated oligonucleotides to solid-phase-immobilized peptides consisting of trifunctional amino acids, such as lysine, arginine, tryptophan, serine threonine, and tyrosine.[83] They have explored different possible protection schemes for introducing these amino acids. Base-labile side chain protecting groups were used as permanent protecting groups throughout the conjugate synthesis. In some cases (where base-labile protection is not possible), temporary protecting groups were used to protect α-amines and the 5′- and 3′-termini of oligonucleotides. For introduction of lysine, they used commercially available N^{α}-Fmoc-N^{ϵ}-Tfa, N^{α}-Boc-N^{ϵ}-Fmoc, and N^{α}-Boc-N^{ϵ}-Tfa protected lysine building blocks. It is reported that, in the case of N^{α}-Boc-N^{ϵ}-Tfa lysine, the incorporation of the first (C-terminus of) lysine residue was more difficult. For introduction of arginine, they prepared a new building block, N^{α}-Boc-Arg(Fmoc)$_2$-OH, as described in Scheme 1. This building block was incorporated into

Scheme 1



peptide sequences. But the oxidation step during oligonucleotide assembly had to be done using tBuOOH (instead of I_2) for better yield of the conjugate. The N^{α}-Boc-N^{in}-(formyl)-Thr-OH afforded better results than N^{in}-unprotected tryp-

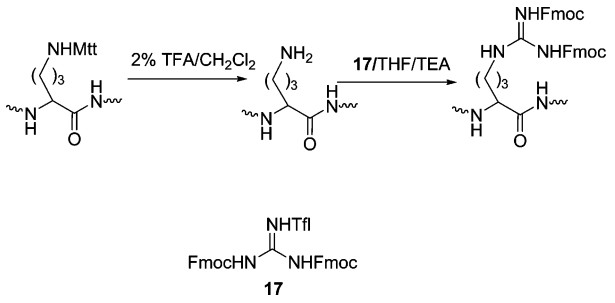
tophans. The hydroxylated amino acids, serine and tyrosine, were successfully incorporated into nucleopeptides using the side chain acetyl (OAc) protected derivatives, namely, N^{α}-Boc-Ser(OAc)-OH and its dicyclohexylammonium salt, Boc-Thr(Ac)-O-HDCHA^{+}.[83]

Also, the tyrosine nucleopeptides are shown to remain stable (irrespective of the fact that the phenolic hydroxyl is a better leaving group) in the presence of free hydroxyl groups. In other words, the phosphate linkage of tyrosine containing nucleopeptides is stable under experimental conditions.

A new protection strategy has been reported for introduction of methionine into nucleopeptides.[84] In this approach, the N^{α}-Fmoc protected sulfoxide derivative of methionine (Met(O))was used as a building block. A number of nucleopeptide hybrids of the type Ac-Lys-Met(O)-Tyr(p3′-dACTAGT)-Pro-OH could be best accomplished by this stepwise solid-phase approach. For example, the above-mentioned nucleopeptide was assembled first by synthesizing the peptide sequence using Fmoc chemistry followed by elongation of the oligonucleotide using the phosphite triester method. Here too, the oxidation was done by tBuOOH instead of I_2. Deprotection and cleavage of the resulting conjugate from the solid support was done by a 1:1 mixture of ammonia and dioxan. Finally, the sulfoxide was reduced to the thioether (methionine) by *N*-methylmercaptoacetamide. However, quantitative amino acid incorporation yields could not always be achieved when the order of assembly of the two moieties was reversed, i.e., by elongating the peptide chain on a resin-linked oligonucleotide in order to avoid exposure of the thioether function to oxidizing conditions.[84]

Azhayev and co-workers have described a strategy for introduction of arginine into POCs[73] wherein they have successfully used Fmoc-Orn(Mtt)-OH as a precursor of the Arg residue. Treatment of the resulting peptide sequences with *N*,*N*′-di-Fmoc-guanidine-*N*′′-triflate (**17**) converts the Orn residues into Arg residues, as shown in Scheme 2.[85]

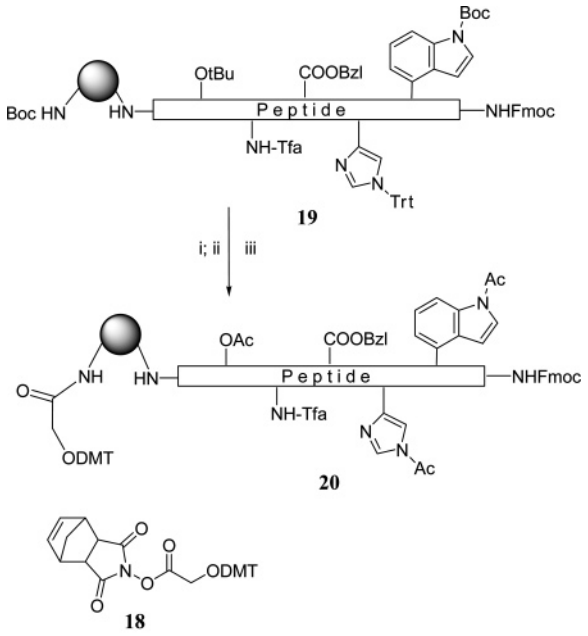
Scheme 2



The guanidine triflate **17** was prepared in a two step reaction. In the first step, the primary amino groups are protected with Fmoc, using Fmoc chloride in the presence of NaOH. The product of the first step is then treated with Tfl_2O in the presence of TFA to yield **17**.

They have also reported modified protocols for introduction of amino acid residues with acid-labile side chain protection.[73] In this modification, after assembling the peptides, the acid-labile side chain protecting groups are deprotected and capped with base-labile acetyl groups, before the oligonucleotide assembly. In a typical method, shown in Scheme 3, the peptide containing acid-labile side chain protecting groups (^{t}Bu for tyroside, Boc for tryptophan, and Trt for histidine) were prepared by Fmoc peptide chemistry, using commercially available N^{α}-Fmoc protected building

Scheme 3



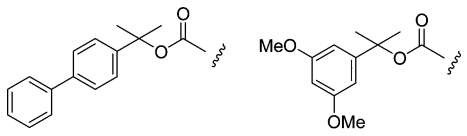
i) 40% TFA; ii)**18**; iii) Ac_2O/*N*-methylimidazole/2,6-lutidine.

blocks. These groups were then deprotected by treating the peptide with 40% TFA (Scheme 3). After conjugation with the linker **18**, the side chains were acetylated to give **20**. The resulting peptide is now ready for assembly of oligonucleotides, by the usual phosphoramidite method.[73] Peptides containing asparagines or tyrosine and lysine residues have been prepared and conjugated to the 3′-terminus of oligonucleotides. Different protection strategies were used for different amino acids.[65] The phenolic hydroxyl group of the tyrosine was protected by mild acid-labile, 2-chlorotrityl-(2-ClTrt) groups; corresponding monomers, Fmoc-Tyr(2-ClTrt)-OH, were obtained from the commercial source. The asparagine residue was incorporated as its amide-unprotected form via use of the Fmoc asparagine pentafluorophenyl ester, to avoid side reactions resulting from carboxylic activations. The β-hydroxy group of the aspartic acid was protected with a base-labile Dmab group (ester). The 2-ClTrt group (of the tyrosine residue) was removed by treatment with 2% TFA-DCM (v/v) for 2 min. Then, the peptide was capped with a base-labile isobutyryl group (by treating the resin bound peptide with isobutyric anhydride). Thus, 2-ClTrt has been used as a transient protecting group for incorporation of the tyrosine residue.

The peptidyl-tRNA analogues containing glutamic acid residues **16** have been synthesized by incorporating side chain allyl esters, to avoid undesired side reactions.[77] After completion of the stepwise synthesis of the conjugates, the allyl group is deprotected by treating with $Pd(PPh_3)_4$ and $PhSiH_3$ in DCM followed by $[NH_4]^{+}[Et_2NCS_2]^{-}$ in DMF. Then the conjugate is cleaved from the solid support using methylamine.

Stromberg and co-workers have recently proposed a uniform protection strategy for the N^{α} of the amino acids and the 5′-hydroxyl of nucleotide building blocks for synthesis of POCs, especially 5′-peptide conjugates of oligonucleotides.[86] In this method, the authors have studied the feasibility of using an acid-labile 2-(biphenyl-4-yl)propan-2-yloxycarbonyl (Bpoc) group (**21a**) and a 2-(3,5-dimethoxy-

phenyl)propan-2-yloxycarbonyl (Ddz) group (**21b**) for N^{α} protection of the amino acid building blocks.



21a **21b**

Several N^{α}-Bpoc and N^{α}-Ddz protected amino acid building blocks are commercially available or can be easily prepared from commercially available reagents. All these groups could be removed easily by treatment with TFA solution (0.2−3%) in a few minutes. The exocyclic amino groups of adenine and cytosine were protected by a benzoyl group, while that of guanine was protected by an isobutyryl group. The histidine side chain was protected by a Dmbz group; this is compatible with Fmoc, Boc, Bpoc, and DMT chemistries and can be easily removed by ammonolysis. The lysine side chain was protected by a Tfa group. This acid-labile urethane and trityl based protection strategy, respectively, for N^{α}- and 5′-OH groups of amino acids and nucleosidic building blocks is shown to be a viable alternative to prepare POCs. In addition, using this strategy, one can synthesize the POCs in "oligo-first−peptide-next" fashion.

2.1.2.2. Protecting Groups Used for Oligonucleotides. Common protecting groups, such as ^{i}Bu and Bzl, have been used for the side chain protection of the nucleobase of the conjugates. For example, the protecting groups ^{i}Bu and Bzl were used to protect G and A or C.[71,82] These groups could be removed by treatment with concentrated aqueous ammonia in dioxane.

In some cases, two different protecting groups have been used for internucleosidic phosphate groups. For example, in the synthesis of peptide-DNA-peptide hybrids,[63] 3′-*O*-(allyl-*N*,*N*-diisopropyl)phosphoramidite building blocks were used for the first four couplings. Then, the peptide was assembled on the 5′-amino modified oligonucleotides using Fmoc chemistry. Finally, the N-terminus of the peptide sequences was elongated with oligonucleotides using 3′-*O*-(2-cyanoethyl-*N*,*N*-diisopropyl)phosphoramidite building blocks. The allyl group was chosen due to its compatibility with Fmoc peptide chemistry. Cleavage from support and deprotection was done with $Pd(0)[PPh_3]_4$/morpholine and a mixture of DMSO/THF/dioxane/0.5 M HCl in a 2:2:2:1 ratio, followed by treatment with concentrated NH_3.

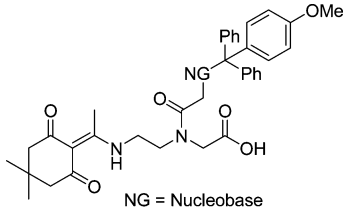
2.1.3. Preparation of Peptide Conjugates of PNA and PMO by Divergent Methods

In principle, solid-phase methods used for conjugation of the unmodified analogues as explained in section 2.2 can be extended for the preparation of peptide conjugates of the oligonucleotide phosphorothioates, 2′-OMe RNA, and LNAs. However, we have yet to see any report describing preparation of LNA−peptide conjugates by sequential solid-phase methods.

PNAs are synthesized using the usual methods of peptide synthesis, and almost any PNA, PNA−peptide, or PNA−small molecule conjugate can be prepared. Even PNAs as long as 40−50 sequences containing consecutively repeated purine bases, and several hairpin PNAs have been synthesized easily using automated synthetic protocols.[87] The ease of preparation and derivatization of PNAs and their enhanced hybridization with DNAs, functional peptides, and small molecules have made them an attractive molecule to be studied in antisence therapeutics. Though PNAs have limited solubility as compared to DNA or RNA, they can be used effectively if simple precautions are taken.[87,88] PNAs are conjugated to the peptides to increase their target binding and uptake by cells or to improve pharmacokinetic properties. The spin labeled PNA−peptide conjugate $(T)_{10}$-AAVALLPAVLLALLA was designed and synthesized by a solid-phase peptide synthesis method on MBHA resin with the Boc strategy.[89] Likewise, PNA−peptide conjugates containing the PNA sequence and D-peptide analogue of IGF1 were assembled on MBHA resin.[90] The peptide part was assembled first, followed by which the PNA was extended as a continuation of the peptide. The conjugate and control sequences were radiolabeled with ^{14}C or fluorescently labeled with fluorescein isothiocyanate. PNA−peptide conjugates containing the peptides derived from the third helix of the homeodomain of *Antennapedia* have been prepared using a solid-phase sequential method.[91] The peptide parts of the conjugates were synthesized first using automated protocols, while the PNA sequences were manually assembled on the same support. The peptide can be attached either at the C- or N-terminus, using a peptide-first or PNA-first strategy. In both cases, mass spectral analysis indicated that the full length conjugate was the major product, and HPLC purification was effective at removing impurities due to failed syntheses.[91]

Conjugation of PNAs with different highly cationic CPPs has been reviewed recently.[37,92,93] The different synthetic approaches used, the properties of the PNA peptide conjugates, and their applications have been described as well.[37] The synthesis of PNA monomers and their subsequent incorporation into PNAs and assembly of PNA−DNA chimeras have also been discussed.[92] However, herein we have summarized some of the recent reports on solid-phase synthesis of PNA−peptide conjugates.

The commercially available PNA monomers can be used for the synthesis of standard PNA oligomers, but their utility for the synthesis of PNA−peptide conjugates is limited, as they do not allow orthogonal synthesis with commercially available amino acid building blocks. Recently, several PNA−peptide conjugates have been synthesized using Dde as the amine protecting group of the PNA monomers using new Dde deprotection conditions that are fully orthogonal to Fmoc chemistry of peptide synthesis.[94−96] The Dde deprotection protocol reported herein is completely compatible with the Mmt protecting group and the nucleobases. The Dde deprotection was done with a solution of $NH_2OH{\cdot}HCl$/imidazole (1/0.75 equiv) in NMP/DMF (5/1) for 1 h. This deprotection condition is reported to be fully orthogonal to other commonly used protecting groups in peptide chemistry, such as Fmoc, Boc, Mmt, t-Bu, and MeO. Novel, Dde/Mmt protected PNA monomers of type **22**, required for the PNA synthesis, have been obtained from an efficient solution-phase synthesis.[95]



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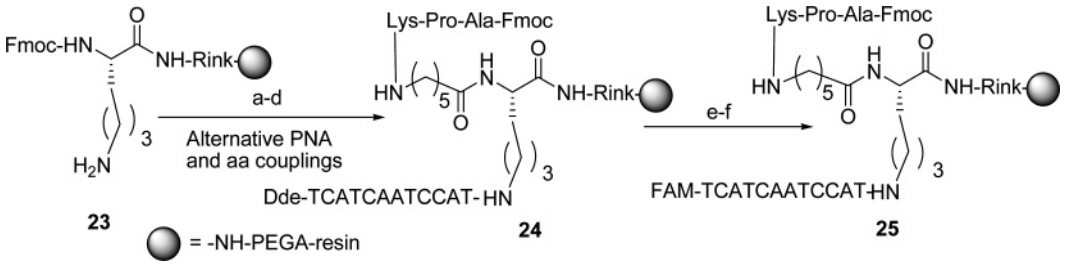
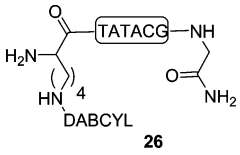
Scheme 4[a]



[a] Conditions: (a) Dde-PNA-OH, PyBOP, NEM in DMF; (b) 20% piperidine in DMF; (c) Fmoc-AA-OH or butyric acid (last step), PyBOP, DIPEA, HOBt in DMF; (d) $NH_2OH{\cdot}HCl$/imidazole; (e) 5(6)-carboxyfluorescein, PyBOP, NEM, in DMF, 15 h; (f) TFA/TIS/CH_2Cl_2.

This method is reported to be much more flexible, whereby elongation of either PNA or peptide part at any stage of the synthesis could be done with ease on a PEGA resin. To demonstrate this flexibility, a conjugate was synthesized by alternating PNA coupling and amino acid coupling steps as shown in Scheme 4 so as to synthesize PNA−peptide conjugates of type **25**. Using this strategy, the authors could successfully prepare fluorescently labeled PNA−peptide conjugates containing tri- and hepta-arginine residues and the short basic Tat 48−57 peptide as examples of cellular penetrating peptides.[96]

It is shown that addition of PPTS and a tertiary amine significantly improved the acylation yields by up to 70%, in carbodiimide mediated coupling reactions of carboxylates to amines in solid-phase synthesis of PNA−peptide conjugates.[97] Using these reaction conditions, the PNA−peptide conjugates labeled with DABCYL chromophores (**26**) could be prepared in good yields.



26

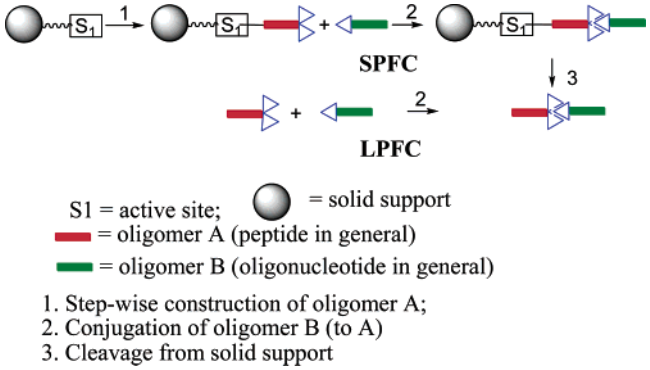
Similarly, several amphiphilic PNA conjugates containing a class of natural peptides, $(Lys)_2$ and $(Glu)_4$ peptides, and *n*-alkanes have been synthesized using Fmoc/Bhoc protected PNA monomers and Fmoc protected PAL-PEG-PS resin with a 0.25 mmol/g loading capacity.[98] Significant changes such as increasing the coupling, capping, and deprotection times and the use of a 5-fold excess of monomer for each coupling are reported to give a better yield of the conjugate.

We have yet to see any report on the solid-phase sequential synthetic method for PMO−peptide conjugate preparation. All the PMO peptide conjugates prepared so far have used liquid-phase fragment conjugation procedure as described in section 2.2.3.

2.2. Fragment Conjugation (Convergent Methods)

The essential steps involved in the fragment conjugation (FC) method for synthesis of POCs are depicted in Chart 3. As can be seen from Chart 3, in the FC method, oligomer A (peptide) and oliogmer B (oligonucleotide) are assembled separately on their own solid supports and they are finally conjugated to each other. A reactive functional group is attached at the desired site of conjugation. The point of conjugation in peptides may be the C- or N-terminus or side chains of the amino acids (Chart 1). In oligonucleotides, the point of conjugation may be the nucleoside, base, or even internucleosidic linkages (Chart 1). If the postsynthetic conjugation is done with one of the oligomers still joined to the solid phase, it is called the solid-phase fragment conjugation (SPFC) method. The conjugates are then cleaved, purified, and characterized. Alternatively, if the conjugation is effected postsynthetically, after complete isolation and purification of the peptides and oligonucleotides, it is called fragment conjugation in the liquid phase (LPFC). The functional groups used so far in such LP and SP conjugations are given in Table 1 (section 1.4). The general reactions used to prepare some of the functional groups are summarized in Figure 4.

Chart 3. Schematic Representation of the SPFC and LPFC Methods



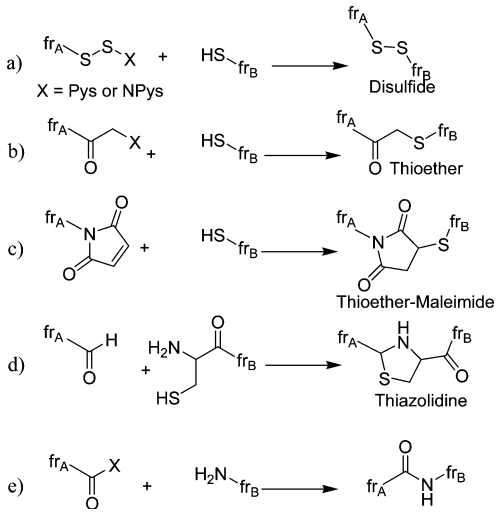


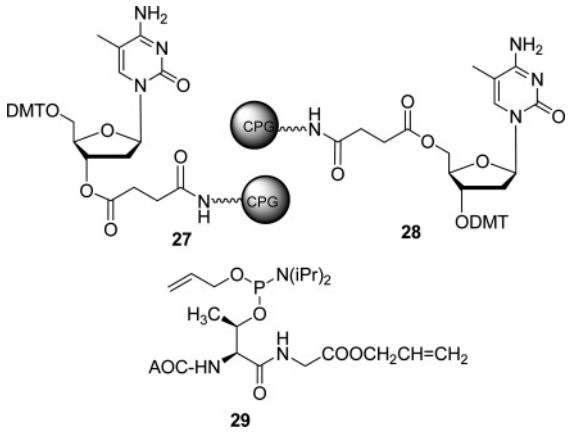
Figure 4. Schematic representation of the most commonly used synthetic protocols to synthesize POCs; fr_A and fr_B can be oligonucleotides or peptides; Py = pyridyl and 4-NO_2-Py = 4-nitropyridyl derivatives.

2.2.1. Solid-Phase Fragment Conjugation (SPFC)

2.2.1.1. Conjugation through the *N*-Acylphosphoramidate Linkage. The *N*-acylphosphoramidates can be prepared

in two steps. The first step is coupling the *N*-phosphitylated carboxamides with alcohol, in the presence of coupling agents such as tetrazole. The second step is subsequent oxidation of the resulting phosphite to phosphate. Alternatively, the *N*-acylphosphoramidates can also be prepared by direct coupling of the phosphatylated primary carboxamide to oligonucleotides. Grandas and co-workers have synthesized a series of (N→5′) peptide−oligonucleotide conjugates by the SPFC method.[99] The peptide and oligonucleotide fragments of these conjugates are linked through *N*-acylphosphoramidate functional groups. In a typical reaction, the side chain protected peptide phosphoramidite Ac-Ser(Ac)-Gly-Asp(OFm)-NH-P(OCNE)N($CHMe_2$)$_2$ (containing N-terminal phosphitylated primary crboxamide) is prepared. This peptide is then coupled to resin bound oligonucleotide, in a procedure similar to the phosphoramidite method. The resulting conjugate is then cleaved by ammonolysis to yield the crude conjugate Ac-Ser-Gly-Asp-NH-p5′CATCAT.[99]

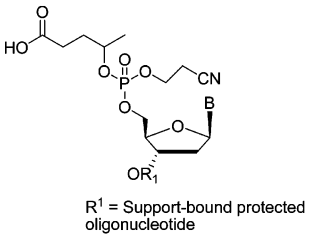
2.2.1.2. Preparation of Nucleopeptides. Hayakawa and co-workers have devised a solid-phase convergent method for the synthesis of nucleopeptides.[100] In this method, the solid-phase immobilized nucleosides **27** and **28** were used

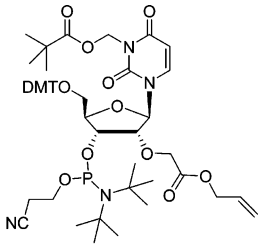


to assemble respectively the 5′-*O*-free and 3′-*O* free oligonucleotides. The internucleosidic phosphite linkages of the oligonucleotide and the C-terminus of the peptides were protected by allyl groups. The nucleoside bases and the N-terminus of the peptide were protected by allyloxycarbonyl groups. The peptide phosphoramidite of type **29** is coupled to the oligonucleotide, which is still coupled to the solid phase. The coupling was effected using benzimidazolium triflate, and the phosphite was oxidized by tBuOOH. Finally, allylic groups are deprotected with a mixture of the tris(dibenzylideneacetone)dipalladium−chloroform complex [$Pd_2(dba)_3$·$CHCl_3$ complex], triphenyl phosphine, and diethylammonium formate in THF (50 °C, 60 min). Then, the conjugates were released from the solid support. Thus, by using this strategy, highly pure conjugates could be obtained in high yields (80−90%).[100]

2.2.1.3. Conjugation through an Amide Linkage. Gait and co-workers have synthesized different types of POCs using SPFC methods.[101−104] They have used a variety of functional groups to link the peptide and oligonucleotide fragments either directly or using a suitable spacer between the fragments. They have used a common strategy, wherein the oligonucleotide is assembled on a solid phase by the usual phosphoramidite method. A modified phosphoramidite building block, which later on becomes the site of conjugation, is included at the desired position of the oligonucleotide sequences. The modified building block carries a reactive functional group, which is then tethered to the C- or N-terminus of the peptides.

Conjugates containing a hydrophobic tetrapeptide, a hydrophobic influenza virus fusion nonapeptide, or a basic octapeptide of the HIV-1 Tat protein coupled to either dT_{12} or a 16-mer anti-Tat ODN have been prepared by this method. It is reported to give better conjugation yield, if the internucleosidic 2-cyanoethyl groups are deprotected prior to peptide coupling. Also, use of a C_{12} spacer between peptide and oligonucleotide is reported to give better yield than without the linker.[101] They have reported successful (5′→C) conjugation of oligonucleotides and peptides wherein the solid supported oligonucleotide carrying the 5′-carboxylate modified building block at its 5′-terminus, **30**, is reacted with various amines and N-termini of small peptides using normal coupling conditions to yield the highly pure conjugates.[103] The building block, **30**, was stable under phosphoramidite coupling conditions but is easily deprotected by mild acidic treatment or by ammonolysis.



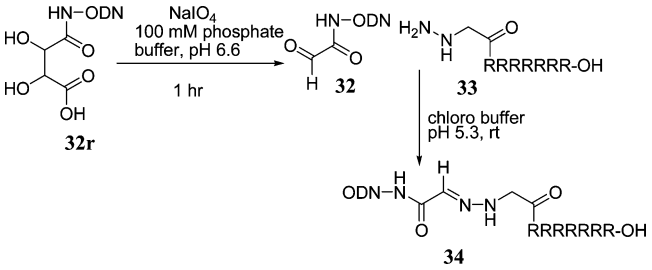


Similarly, they have also reported an efficient method for the synthesis of oligonucleotide 2′-peptide conjugates via amide bond formation on a solid phase.[104] In this method, the oligonucleotides containing a 2′-*O*-carboxymethyl group were obtained by use of a novel uridine 3′-phosphoramidite building block (**31**), where the carboxylic acid moiety was introduced as its allyl ester. This protecting group is stable to the conditions used in solid-phase oligonucleotide synthesis, but it is easily removed by treating with Pd(0) and morpholine. The 2′-*O*-carboxy-methylated oligonucleotides were then efficiently conjugated to peptides through the N-termini of small peptides by a normal peptide coupling procedure, to give the corresponding products in good yields. The products were of high purity.

2.2.1.4. Conjugation through an Oxime Linkage. Oxime is one of the most commonly used linkages to conjugate a peptide and oligonucleotide fragments. This is primarily because oxime formation is an efficient reaction, requires no harsh conditions, and involves highly reactive functional groups, namely, the aldehydes and hydroxylamines. Also, introduction of the aldehyde and hydroxylamine on either fragment is relatively easy. A variety of protecting groups and deprotecting agents are available respectively for masking and demasking these reactive functional groups.[105] Owing to these salient features, different research groups have used the oxime formation for their coupling strategies. The oxime formation protocol has also been used to prepare 5′-conjugates of DNA.[106] In this method, the DNA is assembled first and then it is tethered to a 5′-hydroxylamine nucleophile, immobilized over a solid support. A suitable electrophilic carbonyl compound (peptide) is coupled to form an oxime linkage. The merits of different protection strategies for the hydroxylamine group have also been described therein.[106]

Melnyk and co-workers have recently described a "site specific chemical ligation procedure" with oxime formation as the principle step (Scheme 5).[107] In this method, an

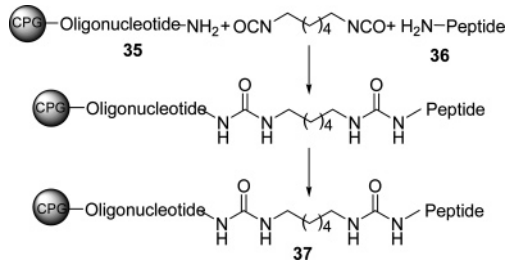
Scheme 5



oligonucleotide carrying a tartamide at the 5′- or 3′-terminus is assembled on a solid phase using phosphoramidite chemistry. At the end of the reaction, the tartaramide is oxidized efficiently to a stable glyoxylyl group (**32**, Scheme 5) by a mild periodate oxidation, in solution.[108] The peptide N-terminal hydrazine (**33**) is then conjugated to yield conjugates of type **34**, in good yield.

2.2.1.5. Conjugation through a Urea Linkage. Fujii and co-workers have described a SPFC method for the synthesis of a series (N→5′) and (N→3′) types of peptide−DNA conjugates containing a ureido moiety as the linking functionality (Scheme 6).[109,110] In this method 5′-amino modified

Scheme 6

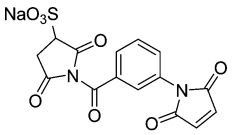


oligonucleotides are assembled on CPG support using the usual methods of oligonucleotide assembly. The N-termini of peptides are linked to these oligonucleotide 5′-amines through a dicyanatoalkane linker.[109] The conjugate was cleaved and deprotected by aqueous ammonia. The DNA−peptide conjugates were isolated in approximately 8−20% yields. By this method, some of the naturally occurring, as well as artificially designed, NLS peptides were covalently linked at the 5′-end, at the 3′-end, and at the middle of oligonucleotide sequences. It is notable that, except lysine, all other amino acids could be used with their side chain unprotected during the coupling procedure. In the case of lysine, the ϵ-amino group of lysine was protected with a trifluoroacetyl group. However, this method required all the peptides to be coupled to β-alanine residue at their N-termini as the reactive nucleophilic moiety, for effective coupling. This method is reported to be versatile enough for the preparation of conjugate molecules such as DNA−sugar, DNA−polyamine, and DNA−lipid conjugates.[110]

2.2.2. Liquid-Phase Fragment Conjugation (LPFC)

The LPFC strategy is also being used to synthesize POCs. In fact, most of the POCs required for biological studies have been prepared by this method. As a first step, in the LPFC method (Chart 3), the peptides and oligonucleotides containing protected, but reactive functional groups, are prepared and purified separately. The pure fragments are subsequently conjugated to each other through the reactive functionality. Some of the reactive functional groups used are given in Table 1. Different types of POCs (given in Chart 1) could be synthesized by this method.

2.2.2.1. Maleimide−thiol Protocol. The maleimide−thiol protocol (Figure 4c) is one of the most commonly used strategies to link the oligonucleotide and peptide analogues. In this strategy, oligonucleotides or peptides containing a maleimide functional group are treated with the peptides or oligonucleotides containing a thiol group, to yield the corresponding conjugates, linked through a maleimido-thioether group. Initially, Tung and co-workers used the maleimide-cysteine protocol to synthesize a series of POCs.[111,112] In this method, the ODNs having either a 3′-amino group or both 3′- and 5′-aminolinker groups were reacted with *N*-iodoacetoxysuccinimide[112] or with **38**[111] to

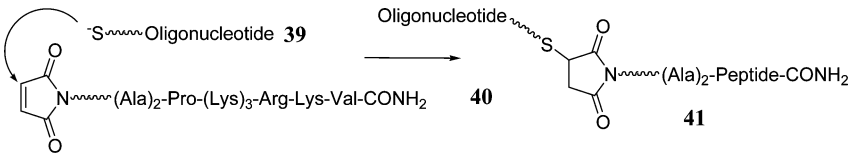


get oligonucleotides containing *N*-iodoacetyl or a maleimide derivative at the respective positions. The resulting oligonucleotides are coupled to peptides containing cysteine to get POCs containing thioether or maleimido-thioether linkages. In a typical procedure, a hexanolamine-linked oligonucleotide 15-mer was treated with maleimide derivative **38**, and the product was purified and further treated with Fmoc-Gly-Gly-His-Cys-NH_2 to give the corresponding POC in 53% overall yield.[111] Using a similar strategy, a series of peptides, consisting of cysteine carboxyamide and a varying number of residues of δ-ornithine coupled through their side-chain amino groups, were linked to ODN 12-mers.[112]

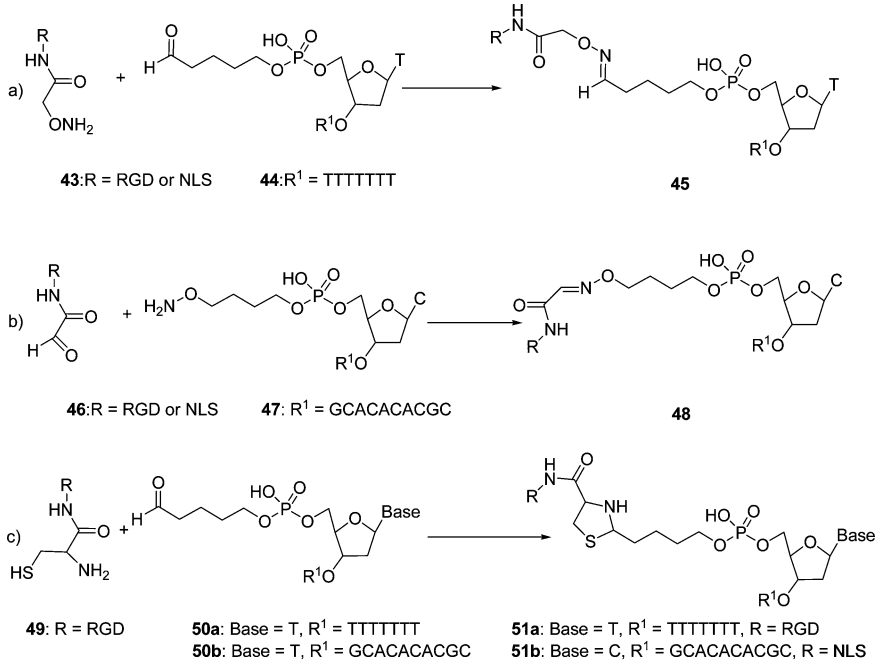
Similarly, Eritja et al. have synthesized a series of oligonucleotide−peptide hybrids[113] using this maleimide-thiol protocol, as shown in Scheme 7, wherein, the oligonucleotides with active thiol function at the 5′-end (**39**) are treated with peptides with a thiol reactive group such as a maleimido group at the N-terminus (**40**) or a cysteine residue to give the corresponding conjugate (**41**). Using this strategy, the authors linked many NLS peptides to antisense oligonucleotides.

The coupling reactions were done at pH 6.0−7.0, and the peptide-to-oligonucleotide molar ratios were 10 to 1. From these experiments, it is concluded that peptide−oligonucleotide hybrids can be easily obtained with both of these methodologies. However, the use of the maleimido approach is highly recommended, as it gives no side products.[113]

Scheme 7

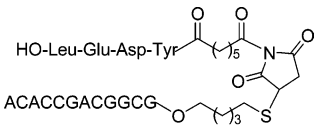


Scheme 8



A reliable method for preparing 5′-thiol oligonucleotides, in good yields, using phosphoramidite chemistry has been reported.[114] In this method, a 6-(tritylthio)hexyl phosphoramidite building block has been synthesized and successfully incorporated at the 5′-termini of the oligonucleotides. These modified oligonucleotides were then conjugated to peptides in high yield (85%) by reacting freshly purified 5′-thiol oligonucleotides with peptide N-terminal maleimides. The purity of thiol phosphoramidite and a manual iodine treatment, after the coupling of the final phosphoramidite, are reported to be important for the success of the reaction.

Mayer's research group has also used this protocol to synthesize a series of POCs.[115] They introduced maleimido functionality into the oligopeptides as described below. The oligopeptides containing α,ϵ-diamino groups are converted into N^{α}-maleimidocapropyl peptides by treatment with ϵ-maleimidocaproic acid-*N*-hydroxysuccinimide ester at pH 6.5 for 1 h.[115] These peptides were subsequently conjugated to the oligonucleotide thiols at neutral or mild basic conditions. In a typical coupling reaction, an N^{α}-maleimidocaproyl-peptide derivative was treated with an oligonucleotide-thiol (carrying a fluorescence moiety) at pH 7.2 to give the corresponding POC of type **42**. The conjugate was obtained



42

in good yields (82%). The thiol group on the oligonucleotide is generated *in situ* by the action of tris(carboxyethyl)-phosphine on an oligonucleotide bearing a disulfide bridge.[115] The free amino group at the 3′-end is available for further appendage; usually a fluorescent moiety is appended at this site for further biological applications.

2.2.2.2. Native Ligation. Stetsenko, Gait, and co-workers have synthesized many POCs using a powerful technique called "*Native ligation*".[116−118] In this method the peptides and oligonucleotides are suitably modified so as to contain cysteine or S-protected thiols or thioesters at the site of ligation, usually the N-terminus. The modified oligomers are then coupled to each other through the modified sites. Thus, peptides derivatized with S-protected N-terminal thioesters were prepared and subsequently conjugated to 5′-cysteinyl oligonucleotides. Stetsenko and Gait have designed and synthesized a number of building blocks and reagents for this purpose.[117,118] For instance, they have prepared peptides containing the *S*-benzylthiosuccinyl moiety at the N-terminus. This peptide was prepared using the standard Fmoc method, and the pentafluorophenyl-*S*-benzylthiosuccinate is coupled in the final step. The resulting peptide was cleaved and side chain deprotected with trifluoroacetic acid. Similarly, 5′-*S*-*tert*-butylsulfenyl-L-cysteinyl functionalized oligonucleotides were prepared using an *O*-*trans*-4-(N^{α}-Fmoc-*S*-*tert*-butylsulfenyl-L-cysteinyl)aminocyclohexyl-*O*-(2-cyanoethyl-*N*,*N*-diisopropyl)phosphoramidite building block in the final coupling step in the standard phosphoramidite method. The oligonucleotide was deprotected and cleaved from the solid support by treating with aqueous ammonia solution. These functionalized peptides and oligonucleotides were used without purification for native ligation reactions in aqueous/organic solution. The *tert*-butylsulfenyl group was removed *in situ* by treating with tris(2-carboxyethyl)phosphine; and thiophenol was used as conjugation enhancer. A range of POCs were prepared by this route.

2.2.2.3. Conjugation through Oxime, Thiazolidine Formation, and Related Reactions. Dumy and co-workers have described a convergent strategy for the synthesis of POCs utilizing chemoselective ligation of peptide to oligonucleotide through oxime and thiazolidine formation (Scheme 8).[119−122] The conjugation through oxime formation was performed by treating an oxyamine containing oligonucleotide or peptide with an aldehyde containing peptide or oligonucleotide (Scheme 8a). Ligation by thiazolidine formation was achieved by treating a peptide containing a cysteine residue with an oligonucleotide containing an aldehyde function (Scheme 8b). In both approaches, the conjugates were obtained in good yields without the need for a protection

strategy and under mild aqueous conditions. Moreover, the oxime ligation was useful for directly conjugating duplex oligonucleotides with suitable peptides.

Using this chemoselective ligation strategy, the authors could successfully synthesize POCs containing RGD peptides linked to oligonucleotides through oxime functionalities (for e.g. **45** in Scheme 8a).[119] The conjugation required slightly acidic (pH 5) conditions and a slight excess of peptide-oxyamine (5 equiv) compared to oligonucleotide-aldehyde. Similarly, they have also prepared POCs with NLS peptide with the same protocol in 50% isolated yields. The oxyamine-containing oligonucleotide and the aldehyde-containing peptides are conjugated to get the POC with glyoxylic oxime linkages (e.g., **48** in Scheme 8b) under the same reaction conditions. The products were purified by RP-HPLC and characterized by ESI-MS. In both cases, the yields were close to 50% after purification by HPLC. The oxime linkage was shown to be stable in phosphate buffers of pH 4 and pH 7 up to 72 h at 37 °C. Oxime bond formation using these two strategies permits rapid and clean preparation of POCs. The coupling of an oligonucleotide-oxyamine to a peptide-aldehyde is less convenient to work up, as the oligonucleotide-oxyamines are very often prone to react with traces of carbonyl compounds present in HPLC solvents. Therefore, the first approach is recommended for the synthesis of oxime conjugates. Similarly, treatment of RGD peptides of type **49** (Scheme 8c) with 4 equiv of 8-mers of type **50** (Scheme 8c), in sodium acetate buffer of pH 5.4 at room temperature, led to formation of POCs of type **51a**, where the peptide and oligonucleotide fragments are connected by the thiazolidine linkage. The reaction was very rapid ($t_{1/2}$ < 15 min) and selective. The same procedure was then applied for the conjugation of NLS peptides too (**51b**; Scheme 8). The products were purified by HPLC and characterized by ESI-MS.[119]

The same research group has recently reported a new method for preparation of oligonucleotides carrying an aldehyde moiety at the 3′-end from the corresponding 3′-1,2-diol precursor.[120] These 3′-aldehyde modified oligonucleotides were successfully coupled to peptides bearing a cysteine residue, forming a thiazolidine linkage.

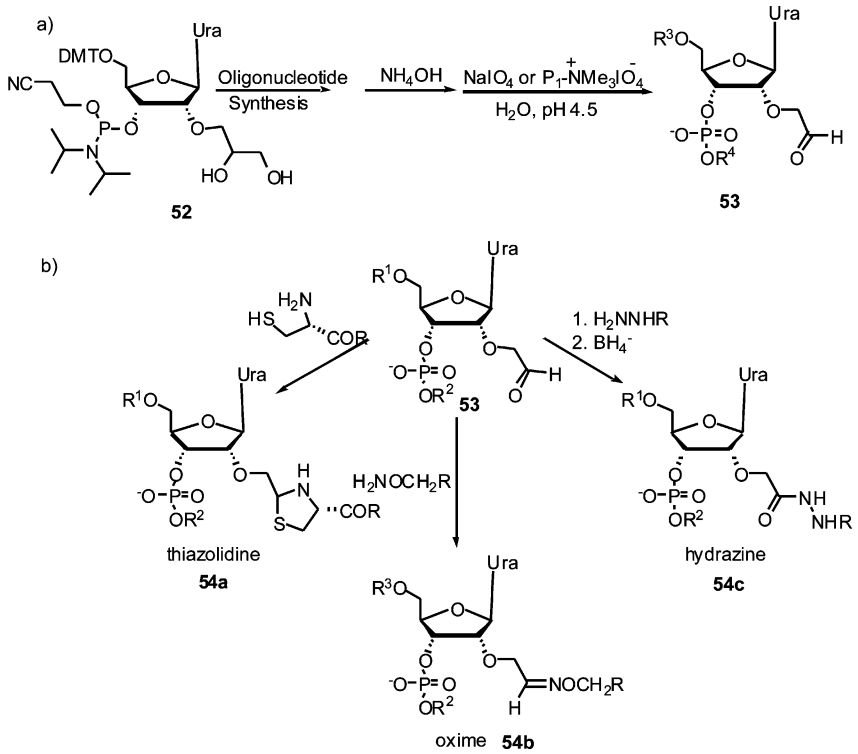
Similarly, these oligonucleotides were also coupled successfully to aminooxy derived peptides, forming oxime linkages. The conjugation reaction was very efficient and selective, thereby allowing the preparation of 3′-peptide conjugated oligonucleotides in good yield. The conjugation was achieved in aqueous solution without using any protection strategy. Moreover, the present approach neither requires the use of peptide in excess nor changes the hybridization properties of the conjugates.[120] Also, it is shown that, using bifunctional ODNs, it is possible to simultaneously conjugate peptides (or carbohydrates) at the 3′- and 5′-ends of ODNs efficiently by this chemoselective oxime bond formation. This method is a single step procedure and can be effected without any protection strategy and under mild acidic conditions. In a typical example, an oligonucleotide carrying aldehyde functions at the 3′- and 5′-ends was conjugated with an oxyamine containing peptide and a mono- and disaccharide. The conjugates were obtained in high yield.[121] Similarly selected sequences of ODNs were conjugated efficiently with distamycin (a minor groove binding polyamide) based peptides containing reactive cysteine and oxyamine functionalities at the C-terminus.[122,123] The conjugation was performed easily within 30−60 min, using individual modified oligonucleotide stretches having sequences of 5′-d(GCTTTTTTCG)-3′, 5′-d(GCTATATACG)-3′, and 5′-d(AGCGCGCGCA)-3′ using oxime and thiazolidine linkages. These distamycin-like polyamide−ODN conjugates were then converted into the corresponding DNA duplexes using complementary oligonucleotide sequences. The major advantage of this ligation technique is that it requires neither a coupling reagent nor chemical manipulations. It requires just mixing the two fragments, namely, the oxyamine and the aldehyde containing oligomers.

Similarly, Gait and co-workers have synthesized a series of 2′-deoxyoligonucleotides and 2′-OMe RNAs carrying one or more 2′-aldehyde groups (Scheme 9a).[124,125] These oligonucleotides were successfully coupled to one or more peptides containing an N-terminal cysteine, aminooxy, or hydrazide group to give corresponding peptide−oligonucleotide conjugates in good yields (Scheme 9b).[124] This facile conjugation method allows specific coupling of unprotected oligonucleotides containing aldehyde groups to unprotected N-terminally modified peptides and other small molecules, in aqueous conditions. Using this strategy, a 12-mer, 2′-OMe RNA, complementary to the HIV-1 TAR RNA stem-loop was conjugated to copies of an 8-mer model laminin peptide.[124] The TAR RNA binding was not affected much upon conjugation. But, the *in vitro* HIV-1 inhibition activity of the conjugate is similar to that of the unconjugated 2′-OMe RNA.

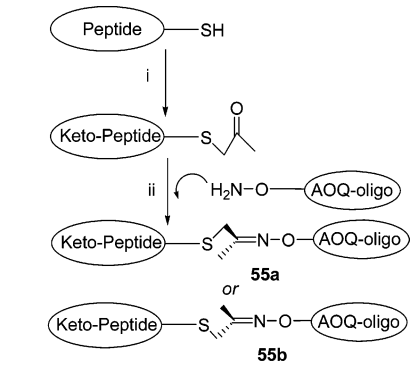
The authors have used 2′-*O*-(2,3-dihydroxypropyl)uridine (**52**) as a suitable precursor of the 2′-aldehyde group. The hydroxyl functions of the 1,2-diol in the modified residue were protected by benzoyl groups, which can be removed during standard oligonucleotide deprotection with ammonia solution. The oligonucleotides containing 2′-aldehydes (**53**) were generated from **52** and subsequently conjugated singly or multiply with unprotected peptides containing different functional groups by nucleophilic addition−elimination reactions as shown in Scheme 9.[126] This strategy is proved to be versatile enough to synthesize POC with oxime, thiazolidine and hydrazone functional groups. In general, the coupling reactions were carried out at pH 4.0−4.5 for thiazolidine and pH 4.5−5.0 for oxime and hydrazone formation. This method proved to be advantageous in many respects. First, it requires no special protection strategy to oligonucleotides, and it requires mild reaction conditions. The reaction is highly specific. Moreover, more than one conjugate can be linked at desired sequences of the oligonucleotide just by coupling more than one 2′-aldehyde modified nucleoside at desired positions. Above all, the conjugation route did not affect the biological property (binding to RNA structures) of the oligonucleotide significantly. In addition, this method does not require use of a large excess of peptide reagent. The hydrazone linkages may be conveniently reduced to hydrazine linkages by use of a solid-supported borohydride reagent. Further, the 5′- and 3′-termini of the oligonucleotide remain free for attachment of other labels, such as fluorescent or radioactive elements, or as substrate for enzyme-catalyzed transformations. The peptide−oligonucleotide conjugates thus synthesized were found to bind to complementary RNA targets with equally high or better affinity than the corresponding unmodified, unconjugated oligonucleotides. Therefore, this 2′-aldehyde conjugation route is believed to be suitable for general use in the design of conjugates of antisense oligonucleotide analogues against RNA targets.

A two step procedure for the preparation of a 2′-*O*-carboxymethyl derivative has also been reported by the same research group[127] wherein the fully deprotected oligonucle-

Scheme 9



Scheme 10



i) $BrCH_2COCH_3$ and 50% CH_3CN/H_2O for 1 h. at rt.
ii) 50% CH_3CN/H_2O for 1 h. at rt.

otide 1,2-diol was oxidized by periodate to the corresponding 2′-aldehyde, which on treatment with sodium chlorite gave the 2′-*O*-carboxymethyl derivative. These 2′-*O*-carboxymethyl oligonucleotides were then efficiently coupled to a number of amino acid derivatives or short peptides to afford novel 2′-conjugates of high purity in good yield. The conjugation was carried out in aqueous solution in the presence of a water-soluble carbodiimide. Thus, using these oligonucleotides, one can prepare POCs containing an amide linkage between the 2′-carboxy group of a modified oligonucleotide and the amino terminus of a peptide.

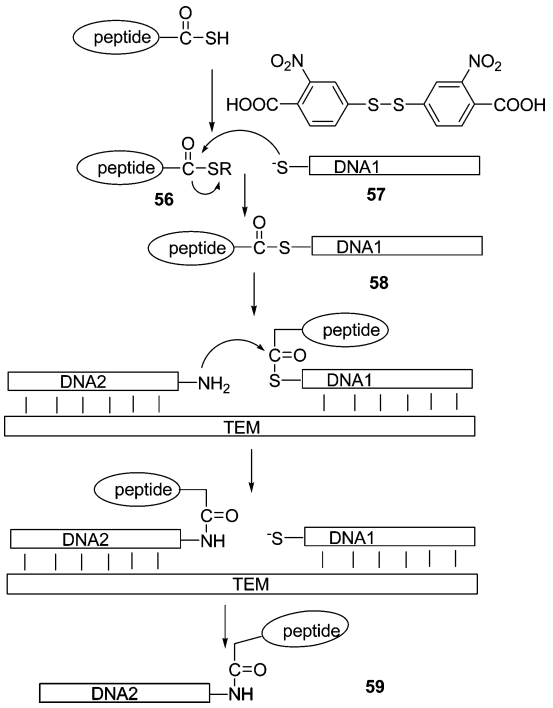
Hama and Miller have recently reported that oligo-2′-OMe RNA conjugated with 4-(2-aminooxyethoxy)-2-(ethylureido)-quinoline (AOQ) and 4-ethoxy-2-(ethylureido)quinoline (EOQ) showed enhanced binding to the HIV-1 TAR RNA targets.[128] They have reported a procedure for conjugation of oligonucleotides containing AOQ and EOQ moieties with keto peptides as shown in Scheme 10.[128] The 5′-termini of the oligonucleotides are tethered with a reactive aminooxy moiety. The modified oligonucleotides were then reacted readily with peptides containing aldehyde and ketone groups, to form stable oxime derivatives, **55a** or **55b** (Scheme 10). This strategy was used to couple an AOQ-oligomer with leupeptin, a tripeptide containing a C-terminal aldehyde group.

The oligonucleotides containing AOQ and EOQ were prepared using modified phosphoramidite building blocks in solid-phase chemistry. The keto-peptide used in this methodology was prepared by treating the peptides containing a cysteine residue with bromoacetone as shown in Scheme 10. Using this strategy, the AOQ containing oligonucleotides were also conjugated to peptides containing RGD residues and a derivative of HIV-Tat peptide having a C-terminal cysteine. It may be noted that the ketone functionality could be introduced anywhere in a peptide containing a cysteine residue.

2.2.2.4. Conjugation through Amide Formation. There are quite a number of reports wherein the fragments of POCs are linked by means of amide bonds (Figure 4e). The amide bond formation between oligomer A (containing a carboxylic acid or thioester) and oligomer B (containing primary amines) is discussed below.

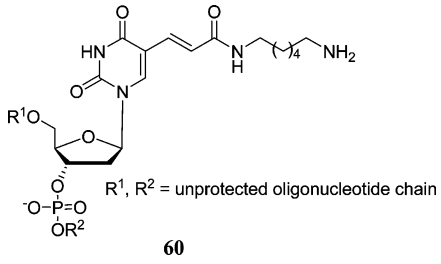
Joyce and co-workers have developed a template directed chemical ligation strategy for trans-conjugation of peptide from one oligonucleotide to another as shown in Scheme 11.[129] In this strategy, first a thioester linked DNA1-peptide conjugate (**58**) is prepared by treating the DNA1-thiol (**57**) with activated peptide C-thioester (**56**). The DNA1 of the intermediate, **58**, binds to a complementary oligonucleotide template (TEM), placing the peptide in close proximity to an adjacent template-bound DNA (DNA2) that terminates in a 3′-amine. The ensuing reaction results in the efficient formation of an amide-linked DNA2-peptide conjugate (**59**). Thus, the oligonucleotide template, TEM, has been used to direct the ligation of peptides to oligonucleotides via a highly stable amide linkage (as in **59**). The ligation reaction is sequence-specific, allowing the simultaneous ligation of multiple oligonucleotide−peptide pairs.

Scheme 11



Similarly, protected ODNs containing 3′-terminal alkylamines were efficiently coupled to C-termini of peptides (and other carboxylic acids) in a convergent manner to yield the corresponding POCs.[130] This conjugation reaction requires extremely mild conditions and does not require a large excess of conjugate substrate. Moreover, the conjugates were obtained in high isolated yields (83−99%).

In some POCs the amino groups of the modified nucleobase have been linked to peptides through different functionalities. For example, various amino acids and dipeptides have been conjugated to modified deoxyoligonucleotides containing the modified thymine **60**.[131] The C-5 position of

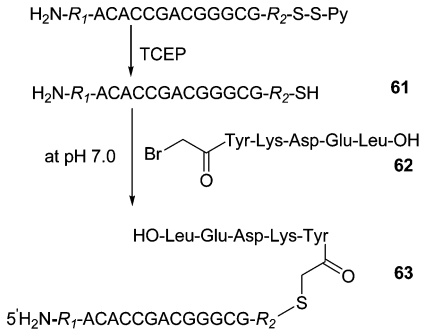


60

the pyrimidine (thymine) bears a reactive amino group. Oligonucleotides containing the modified thymidine nucleotide were prepared and purified by using solid-phase methods. This amino modified oligonucleotide was then coupled to fully or partially protected amino acids or peptides through their C-terminal carboxylic acids, in 50% DMF at pH 7.0. It may be noted that the oligonucleotide and peptide fragments of the conjugates are separated by a spacer arm of 10 atoms. It is reported that the conjugation yield is low for bulky amino acid side chains. However, using relatively high concentrations of EDC, the conjugation yield could be improved remarkably and complete conjugation can be achieved within a few hours. Among four different coupling agents used, EDC was found to have the best coupling efficiency. By this method, they conjugated the dipeptides Ac-His-Gly and Ac-His-Ala with GGT*GG in 69% and 66% yields (where T* represents the building block **60**).[131]

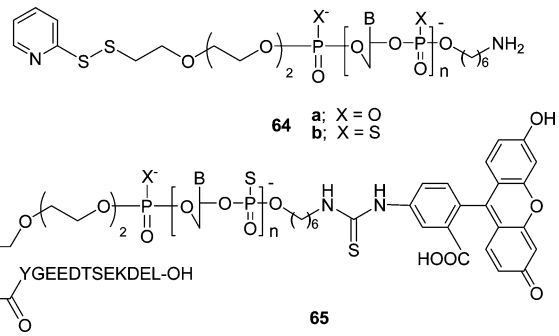
2.2.2.5. Conjugation through a Thioether Linkage. The oligonucleotide thiols have been successfully conjugated to N-terminal-α-bromoacetyl peptides or maleimide-containing peptides (as shown in Scheme 6), in a single step coupling reaction. The oligonucleotide and peptide fragments of the resulting conjugates are linked through a thioether bond. For example, the 3′-amino and 5′-thiol modified dodecanucleotide (**61**) is treated with the (bromoacetyl)pentapeptide (**62**) to yield the corresponding conjugates (**63**) in good yields (Scheme 12).[132] It should be noted that both the peptide and

Scheme 12



the oligonucleotide were readily soluble in the reaction buffers. The optimum reaction condition was found to be 5 h in buffer solution of pH 7.0. Using this method, the authors could synthesize KDEL conjugates of oligonucleotides. The oligonucleotides of these POCs are complementary to the translation initiation region of the gag mRNA of HIV. These thioester linked POCs were found to show higher anti-HIV activity than the peptide-free oligonucleotides.[132]

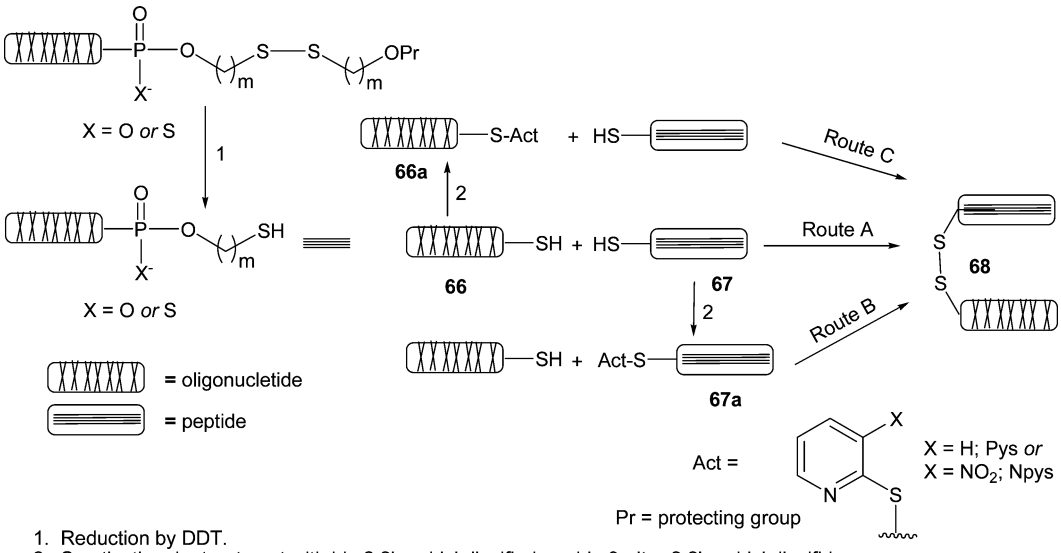
The same research group has recently reported a modified procedure for synthesis of bifunctional oligonucleotides of type **64** and their subsequent conjugation to peptides through



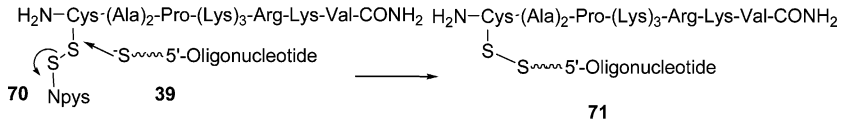
a thioether linkage.[133] The oligonucleotide **64a** and its phosphorothioate analogue **64b** were prepared by the usual phosphoramidite method. A 3′-amino modified phosphoramidite building block was used for attachment of the fluorescent moiety. Then, a linker containing the thioacetyl moiety was manually coupled in two steps by first adding its phosphoramidite derivative in the presence of tetrazole, followed by either oxidation (in the case of phosphate) or sulfurization (in the case of phosphorothioates) to afford the bis-derivatized oligonucleotide bound to the support.

The oligonucleotides were deprotected using a new procedure in which a mixture of 2,2′-dithiodipyridine and concentrated aqueous ammonia, in the presence of phenol and methanol, was used. The phosphate groups and the amino

Scheme 13



Scheme 14

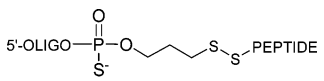


functions of the nucleobases are also deprotected simultaneously. In addition, the 5′-terminal S-acetyl function is transformed into a dithiopyridyl group.

The 3′-fluoresceinylated (5′→C) peptide−oligonucleotide conjugate **65** was prepared from **64** in a three step procedure. First, the 3′-amino group was reacted with fluorescein isothiocyanate to yield a fluoresceinylated oligonucleotide. Then, the 5′-dithiopyridyl group was quantitatively reduced to give a free thiol group. Finally, the resulting 5′-thiol derivatized oligonucleotide was conjugated to the N-terminal-α-bromoacetyl derivative of KDEL signal peptides.

2.2.2.6. Conjugation through a Disulfide Linkage. In addition to thioether and thioester linkages, the disulfide linkage is also more frequently used in oligonucleotide peptide conjugation chemistry.[59,134] Methods used to synthesize disulfide cross-linked POCs are as summarized in Scheme 13. Route A involves direct coupling of peptide and oligonucleotide thiols using suitable reducing agents. On the other hand, routes B and C involve activation of either a peptide or oligonucleotide fragment before coupling with another fragment.

Conjugates of type **69** have been prepared by following the above three different routes. A comparison of the yields



69

revealed that route B, where an activated peptide is coupled to an oligonucleotide thiol, gave the highest yield, followed by route C (activated oligonucleotide coupled to a peptide thiol), and route A gave a poor yield.[135] In fact, the first POC reported was obtained by following route C.[113] In this method, the thiol group of the peptide containing the N-terminal cysteine residue is activated by a 3-nitro-2-pyridinesulfenyl (Npys) group. This peptide (**70**) is coupled to oligonucleotide 5′-thiol (**39**), in the presence of TEAA, to get the corresponding disulfide linked conjugate (**71**) as shown in Scheme 14.

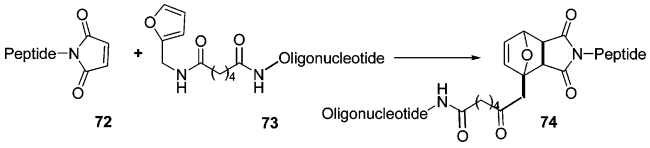
In most cases, the peptide thiol is prepared by introduction of the cysteine residue at the C-terminus or the mercaptopropionic acid residue at the N-terminus. The oligonucleotide thiols are obtained by attaching an O-protected mercaptoproponal phosphoramidite building block followed by reducing with DTT (see Scheme 13). Oligonucleotide and peptide thiols are activated by treatment with 2,2′-dipyridyldisulfide (to form Pys derivatives) or 3-nitro-2,2′-dipyridyldisulfide (to form Npys) in TEAA buffer, in ethanol solvent. The coupling is effected using the disulfide reducing agent, DTT, in TEAA buffer. Simple peptide conjugates of oligonucleotides can be prepared in high yields and purified by RP-HPLC.[116] But in the case of highly cationic peptides, which tend to form complexes with anionic oligonucleotide phosphate backbones, the reaction mixture becomes highly heterogeneous due to precipitation. These factors reduce the yield considerably.[135−137] In an effort to alleviate this problem, the conjugation is carried out under denaturing conditions, namely, under high salt concentrations (0.4 M KCl)[136] or in the presence of 50%−90% formamide.[48,51,135] Subsequently, the conjugates are purified under denaturing conditions using RP-HPLC. An elaborate account on purification of the POCs by RP-HPLC under denaturing conditions is given in section 3.1. An advantage of route B is that the oligonucleotides which are obtained in smaller amounts require less manipulation prior to conjugation. Using route B, Pandey and co-workers have synthesized a series of peptide−PNA conjugates.[50] In a typical method, PNA (1 equiv) and Npys-peptide (1.3 equiv) were mixed in an NMP−H_2O solvent system, in the presence of sodium acetate buffer (pH 5.0). The solution was vortexed and incubated at 40 °C for 3 h in the dark. The reaction was quenched by addition of 5% degassed aqueous TFA, and the resulting

conjugate was purified by high-performance liquid chromatography. As can be seen in the applications section, the disulfide linkage is one of those most commonly used for preparation of POCs of biological significance.

Recently, an improved procedure for synthesis and purification of a mixmer containing 2′-OMe RNA and LNA has been described. The 5′-terminus of the mixmer is attached to the peptide by means of a disulfide bond, while the 3′-terminus is labeleld with a fluorescein label. The significant point is that they have been prepared under highly denaturing conditions, and the mixmers could be conjugated to a range of CPPs, such as Tat (48−58), penetratin, and R_9F_2 peptides.[48,138] The 2′-OMe/LNA 12-mixmer could be conjugated successfully to either the N- or C-terminus of the peptides in good yields. Also, using similar methodology, a 12-mer 2′-OMe RNA and a 16-mer 2′-OMe RNA-phosphorothioate were conjugated to the above peptides in a similar manner.

2.2.2.7. Conjugation through Diels−Alder Reactions. Recently, Picken has exploited the Diels−Alder reaction between a diene and a dienophile to synthesize the POC adducts as shown in Scheme 15.[139] The peptide was attached

Scheme 15



to a dienophile, while the oligonucleotide was attached to a diene. In a representative example (shown in Scheme 15), 2-furfuryl-$NHCO(CH_2)_4CONH$-oligonucleotide (**73**) was reacted with peptide-maleimide derivative (**72**) to form the Diels−Alder adduct (**74**).

The reaction was carried out under highly polar aqueous conditions at room temperature. Because of the very mild and specific nature of the Diels−Alder reaction, unwanted side reactions are minimal, and the reaction does not pose any solubility problem, as both the peptides and oligonucleotides were water soluble.[139] Also, in one such report, the starting materials have been prepared easily by on-column derivatization of the peptides and oligonucleotides.[157]

2.2.3. Preparation of Peptide Conjugates of PNA and PMO by Convergent Methods

Most of the fragment conjugation (FC) approaches described in section 2.2.2 can be extended for preparation of conjugates of modified oligonucleotide analogues as well. Indeed, some of the illustrations, for example, in section 2.2.2.6, involve PNA−peptide conjugation by FC methods. Owing to the emergence of PNAs and PMOs in antisense techniques, the FC methods of conjugation of peptides to these oligonucleotide analogues are given in this section.

Most of the PNA−peptide conjugates reported so far have been prepared by liquid-phase fragment conjugation methods. The frequently used stragety involves conjugation of a Cys-peptide to a Cys-PNA by means of disulfide-bridge. Alternatively, treatment of a Cys-peptide with a maleimide derivatized PNA oligomer yields a PNA−peptide conjugate with a thioether bond.[140] Novel trifunctional peptide−PNA conjugates such as H-$[(KFF)_3K]$-spacer-$NHCH(CH_2S$-DETA$)C(O)$-(TACTCTTAAA)-NH_2 (spacer = -$NH(CH_2)_4C(O)NHCH_2C(O)$- or -$NH(CH_2CH_2O)_2CH_2C(O)$-; DETA = $H_2N(CH_2)_2NH(CH_2)_2NHC(O)CH_2NHC(O)CH_2$-) have been prepared using a convergent synthetic route.[52] The conjugates were assembled by native chemical ligation of a PNA, containing an N-terminal cysteine, with the C-terminal thioester of the cell-penetrating $(KFF)_3K$ peptide to give H-$[(KFF)_3K]$-spacer-$(PNA)NH_2$. The free cysteine side chain of the hybrid was further functionalized with an RNA-hydrolyzing DETA moiety. Similarly, a novel strategy for the synthesis of bisPNA−peptide conjugates by making use of chemoselective ligation of bisPNA to peptides has been used to accomplish oxime formation between an oxy-amine-containing peptide and a bisPNA-Me ketone (complementary modifications can also be used).[141] This method is reported to be highly efficient, does not require a protection strategy, and can be carried out under mild aqueous conditions. Through this methodology, long peptide sequences can be linked to bisPNA in either C to N or N to C polarity. This approach was exploited to link oligonucleotides, sugars, or other chemical entities to bisPNA.[141] A bifunctional PNA sequence containing reactive groups at either end, namely, an N-terminal thiaproline and a C-terminal thioester, has been prepared by the solid-phase method. This intermediate was shown to be useful in construction of a peptide-PNA-peptide adduct using native ligation.[142]

The key aspects of the synthesis and biological activities of conjugates of PMOs with synthetic peptides have recently been reviewed.[59] Conjugation of PMOs to peptides containing various numbers of arginine residues has been carried out by fragment conjugation methods. In this report, different types of cross-linkers have been used to connect the peptide and PMO fragments. The position of conjugation and the length of the PMO is varied in different conjugates. As a typical example, the R_9F_2C-GMBS-PMO conjugate (**78**) was prepared as shown in Scheme 16.[43] The full-length PMO sequence with acetyl protection at its 3′-end and a piperazine group at the 5′-end (**75**) was prepared using automated methods.[41] Then, the 5- (or 6-) carboxyfluorescein was attached to the 3′-end of the PMO. Then, a bifuncitonal cross-linker, for example, **76**, was linked to the 5′-end of the PMO under suitable conditions. The PMO−linker intermediate (**77**) could be isolated and analyzed well by MALDI-TOF MS and HPLC. The conversion from PMO to PMO-linker was >90%.[43]

The PMO-linker (**77**) was subsequently attached to the peptide Cys-F_2R_9 in sodium phosphate buffer (pH 6.5) under suitable reaction conditions. An excess of the peptide (2:1 molar ratio of peptide to PMO) was added to the PMO−cross-linker solution. The resulting conjugate, **78**, was purified first by ion exchange chromatography to remove unconjugated PMO and then by reversed-phase chromatography. The peptide R_9F_2C is to linked the PMO through a linker by means of a thio(ether)-maleimide bond. The final product was analyzed by MALDI-TOF MS and capillary electrophoresis. However, the analysis revealed that the final product contained about 60% peptide conjugated to the full length PMO, while the remaining part of the sample was composed of conjugates of shorter PMOs, a small amount of unconjugated full length and shorter length PMOs, and about 2% unconjugated peptide.[43] Conjugation of the peptide through similar cross-linkers could be done successfully at the 3′-end of the PMO as well.[41]

2.3. Divergent and Convergent Methods: A Comparison

In general, especially for large scale preparations, the solid-phase method should be an ideal one. The major advantage

Scheme 16

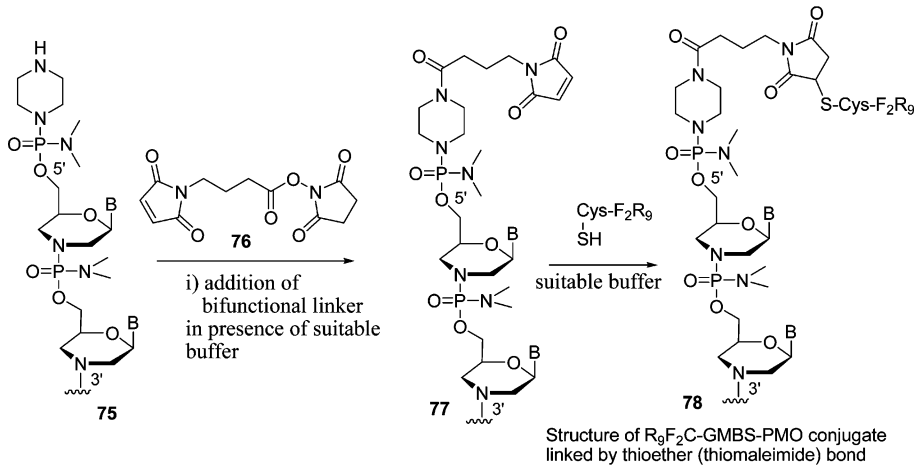


Table 3. Relative Merits and Demerits of Divergent and Convergent Strategies

no.	divergent method	convergent method
1	conjugation is done mostly in the solid phase	conjugation is done in liquid as well as in solid phase
2	the oligonucleotides and peptides are grown on the same solid support	peptides and oligonucleotides are assembled individually and then coupled postsynthetically
3	requires special protection strategies for side chains	in most cases, usual side chain protecting groups are used
4	the nature of the building block plays a vital role in determining the overall yield; only few amino acids, which do not require side chain protection, could be introduced with ease	no such restriction
5	the length of the peptides or the oligonucleotides of the conjugate is also restricted	peptides of any length could be conjugated, provided other conditions, such as solubility are permitted

of solid supported synthesis, as compared to the liquid-phase method, is its less laborious purifications. Most of the side chain products and other soluble impurities are washed away in each stage. Therefore, after cleavage of bioconjugates from solid supports, usually, the conjugates are purified only once to remove the rest of the impurities. The solid phase involves fully automated machine assisted systems from the starting to the ending point. In addition, introduction of several micro array techniques has further increased the potential use of the solid-phase method. On the other hand, in liquid-phase methods (for e.g. in LPFC), the biopolymers are to be purified before and after conjugation. The purification of the conjugate obtained from the liquid phase becomes much more difficult compared to purification of oligonucleotides and peptides, especially if the conjugates contain highly polar amino acids. Some of the merits and demerits of convergent and divergent strategies are given in Table 3.

As mentioned in the Table 3, the solid-phase divergent method becomes tedious for synthesis of long conjugates. In general, the POCs obtained by the solid-phase method contain not more than 10−15 nucleotide or peptide sequences. The type and length of peptide oligonucleotides required for biological studies are mostly prepared by fragment conjugation. The solid-phase method has yet to replace the fragment conjugation methods.

On the other hand, peptides and oligonucleotides of any length could be conjugated using the LPFC method, provided other conditions, such as solubility, can be varied. However, in some cases, e.g. conjugates containing arginine rich peptides,[75] the solution-phase conjugation could not be carried out due to precipitation of peptide (which, in this case, is due to electrostatic interactions). This was overcome by the on-resin fragment conjugation method, wherein the oligonucleotide was adsorbed on an anion-exchange resin and then conjugated by passing a solution of peptide and coupling agents over the resin. Alternatively, the cationic peptides were conjugated successfully to oligonucleotides under denaturing conditions. Vives and Lebleu have reported that the precipitation problem can be reduced considerably by using a 0.4 M potassium chloride solution and 40% acetonitrile; under these conditions they coupled DNAs to highly basic Tat peptides with much success.[136] Similarly, disulfide linked PNA−peptide conjugates were prepared using a formamide−TEA (100:1) strong denaturing system in TEAA buffer.[135] Gait and co-workers have also reported that they could conjugate a variety of modified oligonucleotides, such as oligo LNA, 2′-OMe RNA, and LNA/2′-OMe RNA mixmers, to cationic peptides through a disulfide linkage, using similar conditions, i.e., in the presence of 70% formamide with a TEAA buffer and a 2.5- to 4-fold excess of peptides.[48]

2.4. Biochemical Methods

In addition to the purely chemical methods, the POCs have also been synthesized by biochemical methods. For example, Waldmann and co-workers have synthesized small nucleopeptides by a chemoenzymatic method[74,143] wherein a combination of enzyme-labile and chemical protecting groups were used as C-terminal protecting groups. Advantages of this method are that the biocatalysts used are chemo- and stereoselective and that it requires mild reaction conditions.

Hence, the peptide or phosphate bonds and other protecting groups are not cleaved under the reaction conditions. The authors have used methyl, 2-methoxyethyl, methoxyethoxyethyl, and choline esters as C-terminal protecting groups.[143] The enzymes lipase, butyrylcholine esterase, and papain have been used as deprotecting biocatalyst. Similarly, use of other ligating enzymes in conjugation of peptides to plasmids is discussed in section 4.4.4.

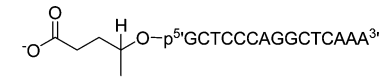
3. Methods of Purification, Analysis, and Characterization

The proper choice of a method for purification of biopolymers such as peptides and oligonucleotides depends on many characteristics of the polymer, such as its molecular weight, composition, charge, polarity, acidity, hydrophobicity, etc. Most often, traditional methods of purification of peptides[144] and oligonucleotides[145] can be extended to purification of POCs as well. However, in some cases, purification of these conjugates necessitates drastic modification of the usual methods adopted for either peptides or oligonucleotides. In this section, we summarize different methods for purification and analysis of POCs. Tung and Stein have recently discussed different methods used for purification, analysis, and characterization of POCs.[28]

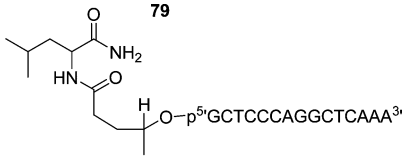
3.1. Chromatographic Methods

Chromatographic methods remain to be one of the most frequently used tools for purification as well as analysis of POCs. Depending upon the complexity of the conjugates, simple HPLC (with normal phase), reversed-phase HPLC (RP-HPLC), and ion exchange (mostly anion exchange) chromatographic (IEC) methods, or a combination of more than one, are used.

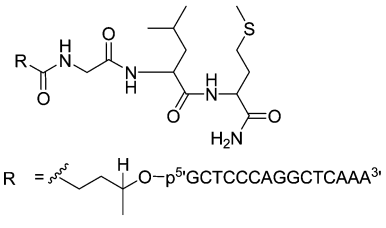
RP-HPLC has been used in most cases to purify the crude POCs. As of now, it is difficult to theoretically predict the chromatographic behavior of a given conjugate with respect to its peptide or oligonucleotide fragments. Oretskaya and co-workers have carried out a systematic study on chromatographic characteristics of a number of oligonucleotide 5′-conjugates (of the type **79**) using the equidistant gradient



79



80



81

method.[146] They synthesized a series of conjugates of amines or short peptides with oligonucleotides containing 5′-terminal carboxyl groups (**79**) by solid-phase chemical synthesis in quantitative yields. The conjugates were synthesized by the SPFC method,[103] wherein the 5′-protected carboxy terminated oligonucleotides were assembled using automated the phosphoramidite protocol. Then, the 5′-free carboxyl group was treated with nucleophilic components (while the oligonucleotide is still attached to the solid phase) in organic medium to get the conjugates, e.g. **80** and **81**, in quantitative yields.

A correlation between the physicochemical parameters and the retention times of the synthesized conjugates in ion pair RP-HPLC was established by the equidistant gradient method.[147] It is based on the principle that the retention time of a given oligonucleotide can be calculated by the nature of the nucleotide and internucleosidic groups and their interactions with different ions on the solid surface of the HPLC column and other conditions such as pH, temperature, etc. For the conjugates **80** and **81**, the theoretically predicted retention times (14.65 min and 15.52 min) were in close conformity with experimental retention times (14.75 min and 15.96 min). However, for oligonucleotide−dipeptide or −tripeptide conjugates, the percentage of error was found to be more. This is due to the increase of complexity of the molecules.

In some cases, the relative mobility of the POC with reference to the oligonucleotide or peptide is predictable. For instance, in a mixture of POCs containing hydrophobic (neutral amino acids) peptides and oligonucleotides, the latter (being more polar) are eluted first.[62,148] But, in a mixture of conjugates containing hydrophilic peptides and oligonucleotides, POCs are eluted almost along with oligonucleotides.[112,114,132]

Purification of POCs with cationic peptides is much more difficult.[124,149] Most of the POCs with cationic peptides have been purified by making use of strong anion exchange (SAX) resins. SAX resins are also used to desalt or exchange the anion of the POC. For example, SAX was used to exchange K^+ or Na^+ countercations with NH_4^+ ions to make separation easy.[72,137] But in the recent past, such POCs (containing cationic peptides) have been purified by ion exchange chromatography under denaturing conditions. Denaturing agents such as salt (high concentrations)[150,151] or formamide[48] have been shown to provide pure conjugates in good isolated yields. For example, a 20-mer phosphorothioate-Ant-20 conjugate, linked by means of a disulfide bond, was purified by IE-HPLC, with a linear gradient of 0.18−1.35 M KBr over 60 min in 0.1 M KH_2PO_4 (pH 6.5), 5 M urea, 25% CH_3CN.[150] Similarly, PNA−cationic peptide conjugates were purified by short path ion exchange chromatography under highly denaturing conditions, using 50% formamide to get the desired conjugate quickly from the reaction mixture containing excess peptide and to prevent precipitation.[48] The conjugates thus purified showed well resolved HPLC chromatograms, and the pure conjugate was by far the major component (conversion >90%). The isolated yield of conjugates was 36−78%. The POCs purified under denaturing conditions were further purified to remove the salt (desalting) and again by RP-HPLC and or dialysis.[151] It is significant to note that the authors have failed to purify these conjugates using an ion exchange column (e.g. DNA Pac PA 100) or a RP-HPLC column, commonly used for oligonucleotide or peptide purifications, due to precipitation and aggregation. In contrast, conjugation and purification, in the presence of formamide as denaturing agent, were successful in all cases. Therefore, this procedure can be recommended for conjugation of a range of cationic or other CPPs to oligonucleotides containing phosphodiester or phosphorothioate backbones.

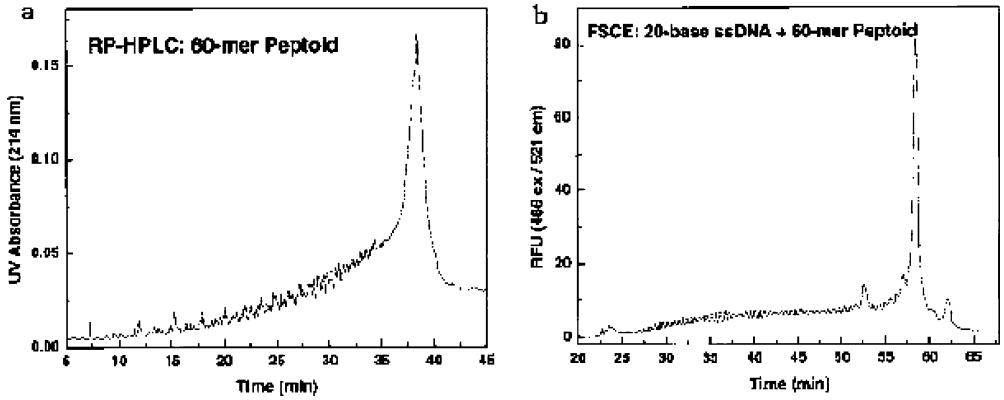


Figure 5. (a) RP-HPLC separation of a crude (unpurified) 60-monomer poly(*N*-methoxyethyl glycine). Conditions: C18 packing (Vydac, 5 mm, 300 Å, 2.1 mm × 250 mm). A linear gradient of 10−60% B in A was run over 50 min at a flow rate of 0.1 mL/min [(solvent A) 0.1% TFA in water; (solvent B) 0.1% TFA in acetonitrile] at 60 °C; peaks were detected at 220 nm. (b) FSCE separation of an unpurified 60-monomer poly(*N*-methoxyethyl glycine) using a 20-base ssDNA "engine". Conditions: buffer, 1X TTE + 7 M urea, pH 8.5; field strength, 300 V/cm; current, ~4 mA; capillary, 100 cm × 25 mm (95.4 effective length). LIF detection, 488 nm excitation, 521 nm emission. Injection: 150 V/cm, 2 s. (Reprinted with permission from ref 159. Copyright 2002 American Chemical Society.)

Overall, RP-HPLC remains to be the most important method for purification of POCs. This method has also been used as an important tool to check the purity and even to identify the POCs. The Fmoc group has been utilized to alter the hydrophobicity of the peptide part, in a way similar to DMT being used for the oligonucleotide part.[111]

3.2. Electrophoresis (PAGE and CE)

Electrophoretic methods have been widely used, mainly for purification as well as analysis of POCs. Many POCs have been purified by polyacrylamide gel electrophoresis (PAGE).[62,149,190] Some conjugates containing cationic peptides were purified by denaturing PAGE.[124] The relative mobility of POCs containing neutral peptides is mainly dependent on the charge/mass ratio of the oligonucleotide part. In the case of POCs containing ionic peptides, the relative mobility is dependent on the net charge to mass ratio of the conjugate. For instance, if the charge of the POC is close to neutral, the POC remains at the base of the gel with no mobility. On the other hand, POCs containing cationic amino acid residues migrate considerably more slowly than the corresponding unconjugated oligonucleotides. For example, the relative mobilities of a series of POCs with varying proportions of arginine-peptides conjugated to a 9-mer oligonucleotide were studied. As expected, the mobility decreased in proportion to the number of arginine residues (positive charges).

In the past few years, capillary electrophoresis (CE) has become increasingly popular as a high-resolution, high-sensitivity technique for the separation and analysis of proteins[152] and DNA.[153−156] The technique has been extended to analyze POCs as well.[62] Barron and co-workers have introduced a novel technique called free-solution conjugate electrophoresis (FSCE), for the high-resolution separation and sensitive detection of uncharged, synthetic PEG.[158] In this technique, the crude polydispersed analyte is conjugated to fluorescently labeled monodispersed DNA, and then, the bioconjugate is separated by free-solution electrophoresis in a long capillary.

The authors have successfully applied this bioconjugate capillary electrophoresis technique to analyze products of the sequential solid-phase synthesis of oligomeric polyamides, namely, poly(N-substituted glycines) or polypeptoids.[159] Compared to RP-HPLC analysis, FSCE analysis of oligomeric peptoids is approximately five times more resolved and separation efficiencies are increased by 150%. Moreover, as laser-induced fluorescence (LIF) is used, the impurities that are not detectable by RP-HPLC analysis are also readily detected and separated. Thus, this method is believed to be an interesting alternative to HPLC for the characterization of chemically produced polyamides. Moreover, with the advent of capillary array electrophoresis (CAE) instruments, now available commercially, FSCE, with a modified conjugation strategy, could allow the products of combinatorial solid-phase peptide synthesis to be analyzed in parallel. The advantage of this method is illustrated by comparing the results of RP-HPLC and FSCE analysis of a set of peptoid−DNA conjugates (Figure 5). A direct application of this technique for a POC has yet to be reported. But the structural similarity of the peptoid−DNA to a POC makes this technique a promising technique to be used in the analysis of POCs.

3.3. Amino Acid Analysis

The peptide part of the PO conjugates is characterized by amino acid analysis (AAA). The correct amino acid composition was typically found for POCs containing different amino acid sequences (except for glycine).[62,70,115,132,160] It had been suspected that glycine might be produced during hydrolysis of the oligonucleotide.[62,70,115,132] To study this unknown glycine,[161] four different nucleosides, namely, deoxyadenosine, deoxyguanosine, and thymidine, were subjected to gas-phase hydrolysis for 48 h in separate experiments. The products were analyzed by ion-pair RP-HPLC with postcolumn fluorometric detection using *o*-phthaldialdehyde/2-mercaptoethanol.[162] The results revealed that only deoxyadenosine hydrolysate showed the glycine peak. This peak was consistently observed in hydrolysates of several different oligonucleotides and POCs. In addition, it was also observed that the commercially available 3′- and 5′-amino linkers produce their own specific peaks on AAA of POC hydrolysates. These specific peaks from deoxyadenosine and the linkers can be used as markers to characterize and quantitate POCs.

3.4. Mass Spectrometry

Mass spectrometry (MS) remains to be a sensitive and efficient technique for analysis and characterization of POCs.

Various MS approaches for characterization of cross-linked protein−nucleic acid conjugates have recently been reviewed.[163] As we were writing this review article, we came across a review article by Banoub et al., on the recent development in characterization of nucleoside, nucleotide, oligonucleotide, and nucleic acids.[164] Thus, many natural POCs are characterized using MS techniques. The molecular ion peaks corresponding to different constituents of POCs provide ample information about the POCs. The sequence, composition of intact peptide−oligonucleotide conjugates, cross-linking yields, and binding stiochiometry, and above all the preferred nucleic acid binding sites, have been established by MS methods. Several new "state-of-the-art" online CE-MS approaches have been designed for the analysis and characterization of POCs and other molecules of biological origin.[165]

Invariably, all the synthetic POCs are characterized by one or another MS method. Electrospray ionization (ESI)[62,132,166,167] or matrix-assisted laser desorption−ionization/time-of-flight (MALDI-TOF)[63,79,80,128] mass spectrometric techniques[168] have been used extensively. It is mainly the oligonucleotide portion of the POCs which determines the behavior of the conjugate in ESI-MS. Another important point to be noted is that the negative ionization method is more suitable for their analysis, especially when the mass of the oligonucleotide part is more than that of the peptide part. The conjugate containing a large number of arginine and lysine residues severely complicates the spectra, as these cationic residues irreversibly bind with salts (during HPLC purification).

In MALDI-TOF-MS methods, carboxylic acids, such as 4-hydroxy-α-cyanocinnamic acid,[82,168] 3-hydroxypicolinic acid,[128] and sinapinic acid,[79] or non-carboxylic acids, such as 2,4,6-trihydroxyacetophenone/diammonium salt,[81] have been used as matrix. Selection of a suitable matrix is generally made according to the mass ratio between peptide and oligonucleotide[168] fragments of the conjugates.

Both ESI-MS and MALDI-MS techniques furnished quite accurate mass values for almost all conjugates, including relatively larger POCs. For POCs with mass values in the range of 9000 Da, the calculated and experimental values were very close with only a few daltons difference. For some POCs, MALDI-TOF-MS is reported to be a bit more sensitive than ESI-MS.[168] Clearly, mass spectral analysis remains to be a standard method for characterizing peptides, oligonucleotides, and POCs.

Auriola and co-workers have recently discussed in detail the use of negative ion ESI-MS and liquid chromatography−mass spectrometric (LC-MS) analysis of various POCs ranging from small T(6)-nucleopeptides to large peptide−oligonucleotide phosphorothioate conjugates and ribozyme−peptide hybrids (3−13 kDa).[169] Using this HPLC-MS interface, in high *m*/*z* range mode, the molecular weight of large compounds could be successfully determined with mass errors of 0.1−3.1 amu. The background noise could be significantly reduced by the use of LC-MS as compared to MS only. The high *m*/*z* range further increases the signal-to-noise (S/N) ratio. This aspect is useful for analyzing molecules larger than 10 kDa, which often produce complex spectra that are uninterpretable below *m*/*z* 2000 (Figure 6). A fair resolution was observed in LC-MS spectra of similar compounds.

Mass spectrometric studies have also been used to study the fate of POCs in biological systems. Large molecular differences between the POC and its constituents, namely, peptides and oligonucleotides, and the high sensitivity of MS have made this a good technique to use to study the *in vitro* stability of POCs. For example, the stability of the chimera of a 19-mer oligonucleotide−phosphodiester covalently linked to the Tat peptide via a disulfide bridge was studied in biological fluid, by using MALDI-TOF spectrometry.[170] The cellular internalization and the intracellular reduction of the disulfide bridge were also studied. The results showed that the chimera was stable in different culture media.

It is worth mentioning some of the recent reports on modification of the MS techniques and use of new instrumental parts to suit the better characterization of biopolymers. Recently, the utility of polyaniline-coated nanoelectrospray emitters for characterization of oligonucleotides and peptides, in the negative ion mode, has been described.[171] It is reported that the emitters are durable enough (for at least 1 h) in the negative ion mode to give a stable signal. The high amount of electric discharge usually associated with negative ion mode nanoelectrospray was not problematic with the polyaniline-coated emitters.

3.5. Spectroscopic Methods (NMR)

In general, NMR methods have been used to analyze the live nucleic acid protein or peptide interactions in solution. Many structural features of the binding segments, such as the conformation and the helicity of the peptide, and the oligonucleotide segments of the POC can be studied by NMR methods. The dynamics of these structural features are also studied using NMR. For example, the binding of the RSG-1.2 peptide to the Rev response element RNA was characterized using multinuclear, multidimensional NMR.[172] The RNA/peptide pair with the strongest affinity was characterized by NOESY experiments.

Recently, Bonora and co-workers have used ^{1}H NMR spectroscopy to characterize chimeric POC, DMT-d(GCAT)-PEG-Gly-L-Phe-Phe-Z, and also some of the intermediates involved in this synthesis.[78] For example, Figure 7 shows a part of the ^{1}H NMR spectra of the conjugate in DMSO-d_6. However, the low solubility of the POCs in organic solvents restricts predominant use of NMR methods of characterization.

3.6. Enzymatic and Other Biochemical Methods

Enzymes of known activity have been used to confirm the proposed structure of the POCs. The oligonucleotide 3′-peptide conjugates are resistant to snake venom phosphodiesterase but are partially hydrolyzed by spleen phosphodiesterase and P1 nuclease.[173] Hence, these enzymes (snake venom phosphodiesterase, spleen phosphodiesterase, and P1 nuclease) are commonly used to confirm the presence or absence of the 3′-conjugation. Such enzymatic cleavage by snake venom phosphodiesterase and alkaline phosphatase has also been used.[71,174,175] Trypsin has also been used to confirm the structure of POCs with cationic peptides.[148] Thus, the structures of many POCs have been confirmed or even ascertained by using them as substrates for enzymes of known activity.

4. Applications

The initial research motive of peptide conjugation was primarily to improve the pharmacokinetic properties of therapeutically valuable oligonucleotides. Different types of peptides were used in the form of either noncovalent

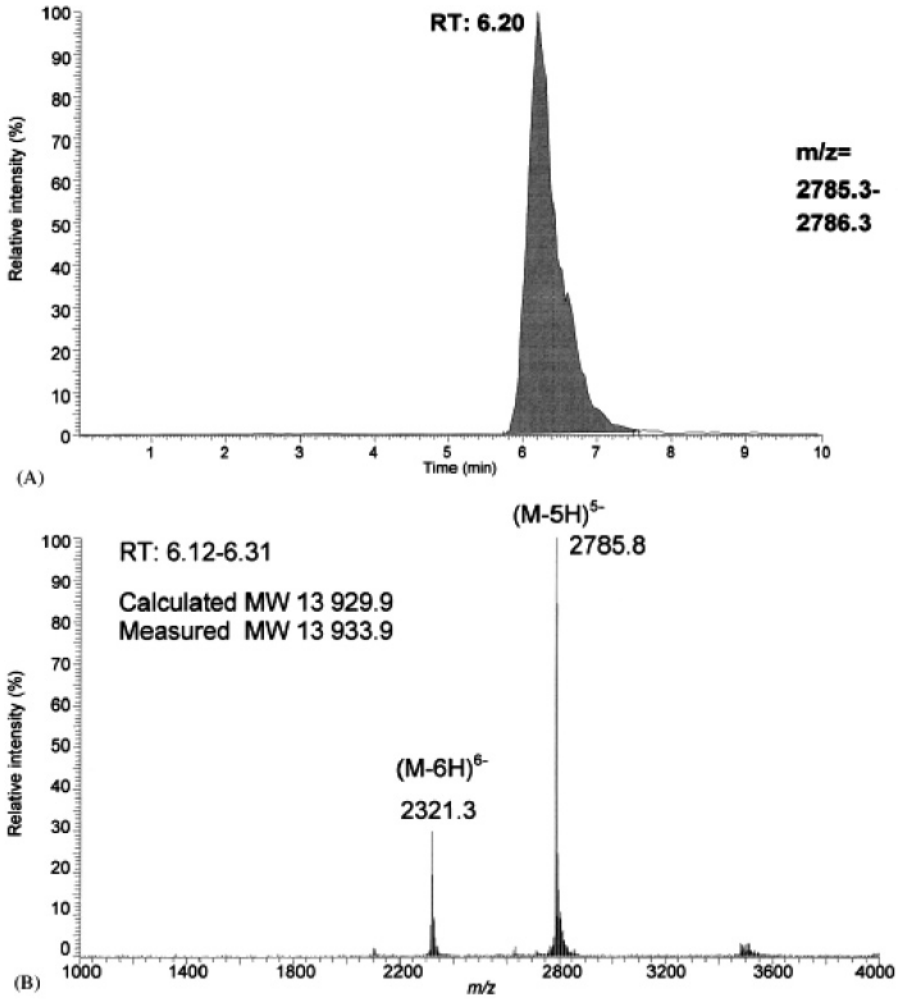


Figure 6. Selected ion chromatogram (A) and full scan negative ion ESI mass spectrum (B) of the ribozyme conjugate Rz-Ant. Injection of 30 pmol (A) TEAA (pH 7; 25 mM) or (B) TEAA (pH 7; 25 mM) in 75% ACN. Gradient: 5−100% B, 5 + 1 min, 100 μL/min. Luna C18 column (50 mm × 2 mm i.d., Phenomenex). (Reprinted with permission from ref 169. Copyright 2003 Elsevier Sciences B.V.)

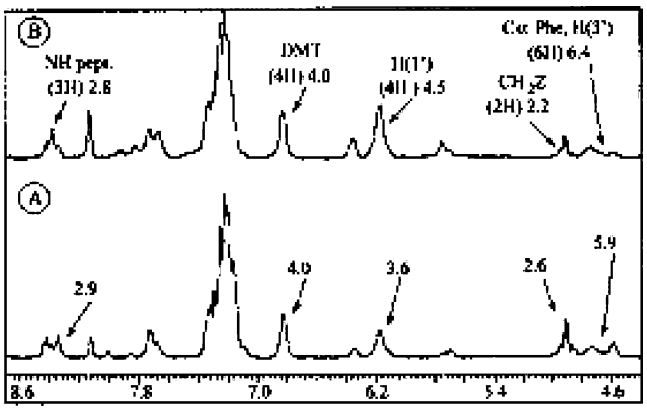


Figure 7. Part of the ^{1}H NMR spectra of the two chimeric PEG conjugates in DMSO-d_6: (A) peptide first; (B) oligonucleotides first. (Reprinted with permission from ref 78. Copyright 2004 Wiley-VCH Verlag GmbH.)

complexes or covalent conjugates to enhance the cellular uptake of different oligonucleotides[13] and other membrane impermeable molecules. The peptides used for cellular delivery can be isolated directly from natural sources or can be synthesized. The most common natural sources of peptides are the peptide vectors derived from viral, bacterial, insect, and mammalian proteins endowed with membrane translocation properties. The peptides used have been classified based on either their source or their functions. Cell penetrating peptides (CPPs) and nuclear localizational signals (NLS) are some of the most commonly used peptides sequences. Peptides play different roles (at different stages), starting from transporting the oligonucleotides across the membrane barrier to enhancing the stability of oligonucleotides against intracellular enzymes and binding to the specific target. The target can be a particular cell or organ or even a specific subcellular organelle. It is significant to note that a given peptide sequence rarely plays a dual role of enhancing the cellular permeability as well as localizations. In the recent past there has been growing interest in using these bioconjugates in many other applications such as models to study mechanistic pathways of internalization of oligonucleotides or to understand the antibiotic activity of oligonucleotides, as primers, aptamers, and so on.

4.1. Therapeutics

Oligonucleotides are hoped to be the medicines of the future to cure many diseases, especially genetically related diseases.[176] The oligonucleotides studied include antisense, antigene, and immunostimulatory siRNA, as discussed below under the respective headings. Most of these studies are aimed at overcoming the poor cellular uptake of naked oligonucleotides so as to realize viable oligonucleotide based drugs. Burgess and co-workers have recently reviewed DNA

Table 4. Sequences of Peptides, Antisense Oligonucleotides, and Conjugates

compd	sequence	ref
82	HOOC-KLALKLALKALKAALKLA-NH_2 (MAP)	178−180
83	FL[a]-5′-GGAGCAGGAAAG-Lys-3′	180
84	FL[a]-5′-GGAGCAGGAAAG-3′-MAP	180
85	5′-CCATGGCAGCGCTGC-LALLAK	76
86	5′-CCATCCCGACCTCGCGCTCC-3′	150
87	H_2N-Ant[b]-S-S-5′-CCATCCCGACCTCGCGCTCC-3′-NH-FL[c]	150
88	H_2N-Tat[d]-S-S-5′-CCATCCCGACCTCGCGCTCC-3′-NH-FL[c]	150

[a] FL = fluorescein. [b] The amino acid sequence of the Ant peptide is NH_2-Ant = NH_2-RQIKIWFQNRRMKWKKGGC (COOH). [c] FL = TAMRA [d] The amino acid sequence of the Ant peptide is NH_2-Tat = NH_2-RKKRRQRRRPPQC(COOH).

based therapeutics and the delivery systems used for them.[177] They have reported that many of them are at different phases of clinical trials.

4.1.1. Antisense Oligonucleotide Peptide Conjugates

There have been a number of review articles on enhancing the cellular uptake of oligonucleotides and their modified analogues, such as PNAs or phosphorothioate oligonucleotides, by different types of peptides.[12−17] Herein we discuss some of the recent studies on enhancement of cellular delivery of antisense oligonucleotides by peptide conjugation. The ability of a MAP peptide sequence (**82**; Table 4) to translocate oligonucleotide phosphorothioates[178,179] and PNAs[180] has been studied. A number of 15-mer antisense phosphorothioate oligonucleotides, directed against the start region of the V2-receptor mRNA, are reported to show enhanced cellular uptake upon conjugation to the MAP sequence **82**.[178,179] A similar observation has also been reported in the case of a 12-mer PNA, 5′-GGAGGAGGAAAG-3′, directed against the mRNA of the noeiceptin/orphanin FQ receptor.[180] This receptor−mRNA had already been proved to be a good target for antisense studies.[181] Incubation of neonatal rat cardiomyocytes and CHO cells with the fluorescein labeled PNA−peptide conjugate (**84**) led to 3- and 8-fold higher intracellular concentrations, respectively.[180] Correspondingly, pretreatment of neonatal rat cardiomyocytes with the PNA−peptide conjugate and the naked PNA slowed the positive chronotropic effect elicited by the neuropeptide nociceptin by 10- and 2-fold, respectively. A more rapid cellular uptake in combination with a lowered re-export and resistance against influences of serum are shown to be the factors behind the enhanced cellular uptake of **84** compared to the naked PNA, **83**.

In addition, the MAP−PNA conjugates were found to be nontoxic in the concentration range up to 1 μM, whereas the corresponding MAP conjugates of phosphorothioate oligonucleotides were toxic at these concentration levels.[180] Thus, MAP is shown to significantly increase the bioavailability and bioactivity of PNA without eliciting enhanced cell toxicity. Similar to delivery peptides, a number of other cell penetrating peptides have also been used successfully to increase cellular uptake and subcellular trafficking of antisense oligonucleotides.[19] In addition, fusogenic and hydrophobic peptides, NLS peptides, and various combinations of these peptide sequences have also been used for effective cellular delivery of oligonucleotides including PNAs.[182]

Peptide sequences derived from different signal peptides are shown to have the ability to perturb the phospholipid model membranes.[183] This property was exploited to deliver antisense oligonucleotides. The antisense oligonucleotide peptide conjugate **85**, which is complementary to the sense sequence of GLUT-1, is reported to show up to 50% inhibition of cell proliferation in HepG-2 and MCF-7 cells as compared to naked oligonucleotides.[76]

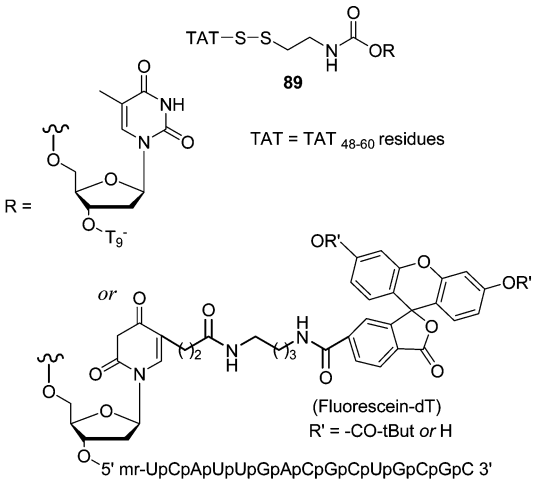
Intracellular delivery and localization of oligonucleotides conjugated to a NLS-peptide sequence have been studied.[184] The conjugates were prepared successfully using the solid-phase fragment condensation method. From the biological experiments, it has been observed that the conjugates are successfully delivered into the nucleus, but their membrane permeability was quite low. However, the conjugates showed increased stability against nuclease digestion. Also, duplexes formed by DNA−peptide conjugates with the target RNA were found to be more stable. Similarly, the hybrid triplexes of dsDNA−DNA conjugates were formed with a higher affinity as compared to those formed by native oligonucleotides.

Many research groups have used Tat peptide of about 12−14 amino acids, derived from the HIV-Tat protein (usually residues 48−60), and Ant, a 16-amino acid sequence, derived from the *Drosophila antenapedia* protein, as delivery peptides to enhance the cellular uptake of antisense oligonucleotides. Recently, these two peptides were conjugated to antisense phosphorothioate oligonucleotides by means of a disulfide linkage (**87** and **88**, Table 4).[150] The conjugates were purified and analyzed by ion-exchange HPLC. These conjugates substantially inhibited cell surface expression of P-glycoprotein at submicromolar concentrations of the conjugates at serum-replete conditions. It is surprising to note that the POCs were more potent in the presence of serum than when used under serum-free conditions. This is in striking contrast to most other approaches for intracellular delivery of nucleic acids. This is mainly because of the relatively modest molecular size of the conjugate. Their good efficacy, even in the presence of serum proteins, makes this type of peptide−antisense oligonucleotide conjugate a better candidate for *in vivo* therapeutic applications.

The same research group has also prepared the Tat and Ant conjugates of various antisense 2′-*O*-methyl-3′-phosphorothioate modified oligonucleotides by means of the LPFC method.[151] The binding and biological properties of the POCs were analyzed. From experimental observations, it is shown that the POCs entered cells over a period of hours and accumulated in cytoplasmic vesicles as well as in the nucleus. The peptide conjugation did not show any adverse effect on the binding affinities as well as the sequence specificities of the oligonucleotides. Thus, it is concluded that conjugation with Ant and Tat enhances oligonucleotide delivery to the nucleus without interfering with the base-pairing function of antisense oligonucleotides.

The 3′-methylphosphonate derivatives of antisense oligo-2′-OMe RNAs are shown to be resistant to nuclease hydrolysis and show high binding affinity to their comple-

mentary sequences of HIV-TAT-TAR regions, under physiological conditions; however, further studies were hampered due to low cellular uptake.[185] Hence, in a recent study, 3′-methylphosphonate modified oligo-2′-OMe RNAs were conjugated to cell penetrating HIV-Tat peptide through the 5′-terminal end of the oligonucleotide. The peptide and oligonucleotide fragments are linked by a disulfide bond (**89**).[137] They could also be prepared by conjugation of a keto



mr-denote 2'-*O*-methyl sugar, and p-denote methylphosphonate linkage

derivative of the Tat peptide to a 5′-AOQ derivatized oligonucleotide as shown in Scheme 10 (section 2.2.2.3). Studies on the cellular uptake of these conjugates by mouse L929 fibroblasts in culture were carried out. From these studies, it is shown that 3′-methylphosphonate modified oligo-2′-OMe RNAs are stable to the 3′-exonuclease activity found in mammalian serum. Due to the high propensity of the Tat-conjugated oligonucleotides to form aggregates, the purification of the conjugate was difficult. Also, their high affinity for glass and plastic surfaces made cellular uptake studies incomplete.[137]

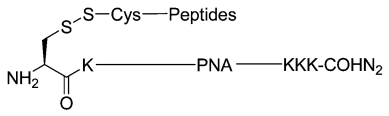
The TAR RNA stem-loop that occurs at the 5′ end of HIV RNA transcripts is an important antiviral target and is the site of interaction of the HIV-1 Tat protein together with host cellular factors. Gait and co-workers have designed and synthesized oligonucleotides of type **90**, composed of 2′-OMe RNA and LNA residues, complementary to HIV-1-TAR RNA, and they studied the effect of peptide (CPPs) conjugation on their ability to block Tat-dependent trans-activation of HIV-1, in a HeLa cells.[48] The conjugates were prepared (as described in Scheme 13) and purified under denaturing conditions. Highly cationic peptides such as Tat (48−58), Penetratin, R_9F_2, etc. have been used in this study. Peptides have been conjugated via their C- as well as N-termini, and the peptide and oligonucleotide fragments of the conjugates are joined by means of disulfide linkages. Some of the POCs used in this study are given in Figure 8.

The cellular uptake characteristics of the CPP conjugates of **90** and two other oligonucleotide analogues, namely, 2′-OMe RNA and 2′-OMe RNA-phosphorothioate (only **90** is shown Figure 8), were studied in detail. From the results, it is shown that none of the naked oligonucleotides enter cells, while some of the conjugates showed weak fluorescence; among them, the C-terminal Tat conjugate of **90** showed a little more noticeable internalization for most cells (Figure 9A).

As the authors felt that the complete charge neutralization of conjugates might be critical for the uptake, peptides with

CPP-90 Peptide-S-S-$(CH_2)_6$-5' -2' -OMe/**LNA**/ **CUC CCA GGC UCA** -3' -Flu
Flu = fluorecein label
Peptide = Penetratin C-NH_2;

91 Flu-Tat-O-PNA-$CONH_2$
Flu = fluorescein label
O = -$NH(CH_2CH_2O)_2$-CH_2CO- (AEEA linkage)



PNA = *c*-CTC CCA GGC TCA GAT C-*n* (**93**)

Figure 8. Structures of peptide conjugates of mixmer **90** and PNA **93**.

additional cationic charges were conjugated to the above **90**−CPP conjugates. For example, the **90** conjugate was conjugated to four additional lysine residues on the N-terminus (K_4). Although they showed substantial cellular uptake, for example, as shown for the K_4−Tat conjugate of **90** (Figure 9B), unfortunately, none of these conjugates showed significant inhibition of trans-activation of HIV-1, as they failed to show nuclear entry. In yet another attempt, corresponding peptides were added to the culture media containing the conjugates, at different concentrations; even in this case, they only showed dose dependent enhanced cyctosolic uptake, but the conjugates failed to enter into the cell nucleus. For example, the cellular uptake of the Tat conjugate of **90**, after addition of 2 equiv of Tat peptide, after 5 h, is shown in Figure 9C. However, Lipofectamine 2000 mediated delivery of naked **90** showed nuclear entry in addition to cytosolic uptake. Thus, it is concluded that the release of the conjugate from the cytosolic vesicles is the limiting step to realize the nuclear entry and, so, trans-activation inhibition of the HIV-1 TAR element.

Based on a series of in-depth studies, the authors conclude that free uptake of the 12-mer OMe/LNA mixmer **90** conjugated to Tat (48−58), Penetratin, and R_9F_2 was observed in the cytosolic compartments of HeLa cells. Also, uptake of the Tat conjugate of **90** was shown to be enhanced by N-terminal addition of four Lys or Arg residues or a second Tat peptide. However, none of the conjugates could enter the nucleus or inhibit trans-activation when delivered freely, but inhibition was observed in the presence of cationic lipids. It is mentioned that the cationic charge of the peptide is not the only factor in mediating cell uptake of the conjugate. It is concluded based on the observation that neither of the C- or N-terminal R_9-conjugates of oligo **90** showed significant uptake into HeLa cells. But R_9F_2, Tat, and Penetratin conjugates of **90** did show better cellular uptake. Hence, it is important that the peptide have additional amino acid residues apart from the residues with cationic charge. The additional residues act either to space the cationic charge more amphipathically or perhaps to add some hydrophobic character to enhance membrane crossing.[48]

Similarly, the 16-mer PNA directed against the HIV-1 TAR RNA stem-loop, present at the 5′-end of HIV RNA transcripts, has been synthesized as a potential antiviral candidate. The cellular uptake characteristic of PNA−CPP conjugates has been investigated.[51] Two different chemical linkages have been used to link the PNA and CPP fragments of the conjugates. One class of conjugates (**91**; Figure 8) are held by means of a short polyether linkage, called AEEA (-$NH(CH_2CH_2O)_2$-CH_2CO-), while the other class of conjugates contain disulfide linkages (**92**; Figure 8). From the biological experiments it is shown that the disulfide linked

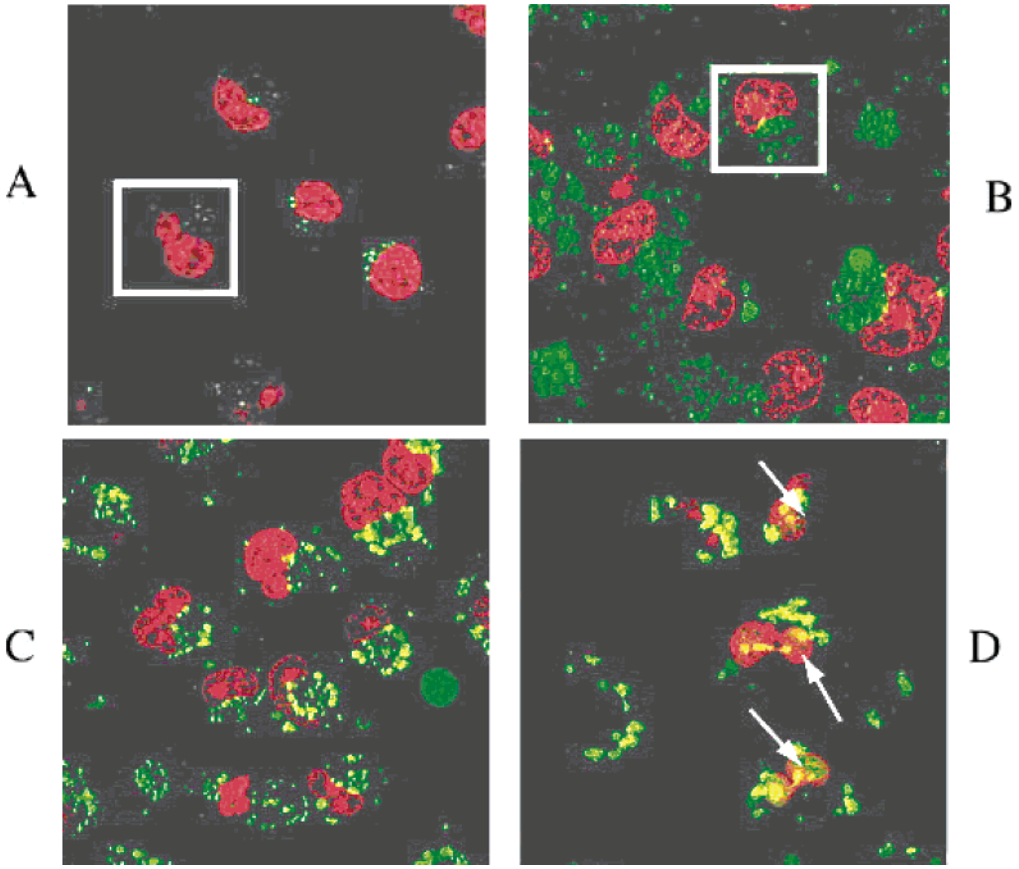


Figure 9. Confocal microscope images of the free uptake of oligonucleotide **90** upon conjugation of different CPPs into HeLa cells; the fluorescein labeled conjugates are green, and the nuclei are stained red. (A) **90** conjugated with the C-terminus of the Tat peptide. (B) **90** conjugated to peptides containing an additional cationic charge, i.e., conjugated with K_4-Tat, after 24 h. (C) Effect of addition of 2 equiv of the Tat peptide on uptake of the Tat-Cys-**90** conjugate, after 5 h. (D) Lipofectamine 2000 delivery of 0.5 mM oligo **90**, 3 h. Note in D the nuclear localization seen (white arrows) in addition to cytosolic uptake. The square in A and B shows a single cell. (Reprinted with permission from ref 48. Copyright 2005 Oxford University Press.)

transportan and a novel chimeric peptide, the R_6-Penetratin conjugate of PNA, showed dose dependent inhibition of Tat-dependent trans-activation in HeLa cells when incubated for 24 h. However, the significant point of this work is that the nature of the linkage between peptide and oligonucleotide fragments does not play a vital role, as it is widely anticipated that the easily reducible disulfide linkage would be preferable as compared to other stable linkages.[50]

In the recent past there have been a few reports on triggered cytosolic release of the conjugates.[51,186,187] In these reports a lysosomotropic reagent, chloroquine, has been added to the culture media to enhance the cellular activity of the conjugates. It is shown that some conjugates (for example, the stably linked Tat peptide−PNA conjugate) showed some activity upon coadministration of chloroquine but no activity in its absence. Similarly, for disulfide linked PNA conjugates of transportan, or chimeric R_6-penetratin conjugates, the maximum efficiency was reached in 6 h upon culturing the cells with 100 μM chloroquine. Previous to this report, it has already been shown by Folini et al.[186] that endosomal release of naked PNAs can be triggered by a photochemical approach. Here it is shown that incubation of a naked PNA targeted to hTERT mRNA in adenocarcinoma cells DU145 for 18 h with a high PNA concentration (10 mM) and a photosensitizer, followed by a 60−80 s fluorescent light treatment, led to a significant reduction in telomerase activity in the cell extracts. This was also confirmed by some microscopy evidence showing fluorescent PNA redistribution into cytosol and the nucleus from endosomal vesicles. But these authors did not test the effect of a similar photochemical treatment on the Tat peptide conjugate of the PNA.[186]

It has recently been shown that the endosomal release of the conjugates can be triggered by coadministration of calcium ions or chloroquine.[187] In this report, stably linked Tat peptide-O-PNA or R_7-PNA conjugates aimed to correct mis-splicing of luciferase mRNA showed respectively 44- and 8-fold increases in activity on using 6 mM Ca^{2+} ($CaCl_2$) and 60−120 mM chloroquine, in the nucleus of HeLa pLuc 705 cells. The mechanism is reported to involve endosomal release, but no such effect was observed for naked PNA.

Tat peptide−PMO conjugates targeting *c-myc* mRNA were prepared.[41] From the biological experiments it is shown that the conjugates successfully down-regulated *c-myc* reporter gene expression with nearly 100% inhibition at an IC_{50} of 25 M. It is significant to note that neither unconjugated PMO nor unconjugated Tat peptide caused antisense activities. However, compared with mechanical mediated delivery, Tat peptide mediated PMO delivery required higher concentrations of PMO (>10 μM) to cause antisense activity and caused some toxicity. Most Tat peptide−PMO conjugates were associated with cell membranes, and internalized conjugates were localized in vesicles, cytosol, and the nucleus. Nonetheless, the Tat peptide significantly enhances delivery of PMO in 100% of cells assayed. Tat peptide mediated delivery is a much simpler procedure to perform than other delivery methods.[41]

Table 5. Effect of the Linker and Linking Chemistry on the Antisense Activity of the R_9F_2C[a] and 705-FL[b] Conjugates[c] [43]

compd	treatment	linker type[d]	linkerspacer (Å)	antisense activityRLU/μg protein (range)
	vehicle control (H_2O)	N/A	N/A	1 (0.1)
94a	R_9F_2C-705-FL	thiomaleimide	6.8	102 (4.9)
94b	R_9F_2C-EMCS-705-FL	thiomaleimide	9.4	141 (4.3)
94c	R_9F_2C-KMUS-705-FL	thiomaleimide	15.7	171 (14.3)
94d	R_9F_2C-SMPB-705-FL	thiomaleimide	11.6	123 (2.1)
94e	R_9F_2C-SMCC-705-FL	thiomaleimide	11.6	86 (1.4)
94f	R_9F_2C-SBAP-705-FL	thioether	6.2	98 (3.2)
94g	R_9F_2C-SPDP-705-FL	disulfide	6.8	109 (2.9)
94h	R_9F_2C-LCSPDP-705-FL	disulfide	15.6	181 (7.8)

[a] The amino acid sequence of the R_9F_2C peptide is NH_2-RRRRRRRRRFFC-$CONH_2$. [b] 705-FL is the antisense PMO with sequence 5′-CCT CTT ACC TCA GTT ACA-3′-fluorescein. This antisense oligomer is targeted to a mutant splice site at nucleotide 705 of human β-globin intron 2. [c] HeLa pLuc705 cells were treated with 10 μM of an indicated conjugate in the serum free medium for 6 h. The antisense activity was indicated by luciferase activity, expressed as RLU/μg protein. Each data point is an average ± range of means from two independent experiments, each consisting of a triplicate sample. [d] The structures of some of the linkers are given in Figure 10.

Similarly, the efficacy and the factor affecting cellular delivery of PMOs by means of complexation as well as covalent conjugation to arginine rich peptides were investigated recently.[41] For this purpose, the authors synthesized peptide conjugates of the target PMOs (e.g., 705-FL) using different linking units as shown in Figure 10. The conjugates

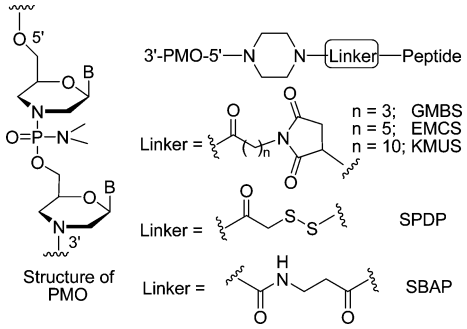


Figure 10. Structure of the PMO-linker−peptide conjugates (**94**) (See Table 5 for details).

were prepared using the fragment conjugation method.[43] For example, the peptide R_9F_2C was conjugated to PMO, 705-FL, through different linkers to yield the conjugates **94a**−**94h** (Table 5). In the case of GMBS, a bifunctional cross-linker containing an amine reactive NHS and sulfhydryl reactive moieties has been used. The 5′-end of the PMO was conjugated to one end of a cross-linker using NHS chemistry. The opposite end of the cross-linker was conjugated to the sulfhydryl group of R_9F_2C by maleimide, bromoacetyl, or pyridyl disulfide chemistry. The biological experiments showed that the PMOs can be successfully delivered to the cell nucleus and cytosol by covalent conjugation rather than complexation of peptides to PMOs. The peptide R_9F_2C was the best suited among the peptides studied (Tat peptide, PTD4, and Ant peptide; see Table 5) to deliver a PMO (705-FL) to its target RNA, resulting in the strongest antisense effect even at low concentrations (in the micromolar range).

The R_9F_2C−PMO conjugates **94a**−**94h** were more effective than the PMO conjugates of the transmembrane transport peptides of the HIV-1 Tat protein, the peptides of the *D. antennapedia* protein, or peptides with fewer arginines. For example, the PMO conjugated to R_9F_2C corrected splicing 4, 14, or 19 times more effectively than the PMO conjugated to Tat, Ant peptides, or PTD4, respectively. The sequences of the Tat peptide, the Ant peptide, and PTD4 are shown as a footnote of Table 5. PMO containing R_9F_2C at the 5′-end had higher antisense activity than the PMO with R_9F_2C at the 3′-end. The culture conditions, such as the cell density type of the medium and the serum concentration, affected the cellular uptake of the R_9F_2C−PMO conjugate. The length of the PMO did not affect the peptide's delivery efficacy, but it affected the antisense activity of the PMO. Thus, the R_9F_2C peptide provided a simple and efficient delivery of PMO to an RNA target. Another significant observation is that the antisense activity was affected by linker length and not by the conjugation chemistry. The antisense activity of the PMO was increased with longer linkers but not affected by the conjugation chemistry (Table 5). An increase of the backbone and side chain flexibility of an arginine-rich peptide or an addition of a hydrophobic moiety onto a peptide has been shown to give better delivery of fluorescein cargo. The higher antisense activity of the conjugates with longer chains of $(CH_2)_n$ moieties is believed to be due to their greater flexibility and/or hydrophobicity, as compared to those with shorter linkers. It is also possible that a longer linker reduces steric interference for PMO binding to the targeted pre-mRNA.

4.1.2. Triplex Forming Oligonucleotide−Peptide Conjugates

Similar to antisense oligonucleotides, triplex-forming oligonucleotides can also be used to modulate gene expression. However, unlike antisense oligonucleotides, the TFOs bind the target dsDNA sequence specifically and most importantly without disturbing the double strand structure of DNAs. TFOs can also be used as a safe delivery vehicle to place a reactive functional group at a specific sequence of the dsDNA. In antigene radiotherapy, TFOs containing radionuclides are used to deliver the radionuclide to the target gene at a specific sequence of the DNA. Then, the high dose of radiation from the radionuclide damages the selected sequences.

Conjugation of peptides to such TFOs is proved to enhance their cellular uptake and target specificity. The ability of ^{125}I labeled TFOs to produce sequence-specific breaks within a target in the human mdr1 gene overexpression in KB-VI tumor cells *in vitro* has been demonstrated well.[188] A TFO with a binding site in intron 14 of the mdr1 gene was labeled with an Auger-electron-emitting radionuclide, ^{125}I. Decay of ^{125}I releases a shower of low energy electrons that produce DNA strand breaks, mostly within 10 bp from the decay site. TFO and TFO conjugated with a NLS peptide were delivered into cells using cationic liposomes. This was done either alone or in the presence of an excess of "ballast" oligonucleotides with an unrelated sequence. Nuclear localization of TFO and survival of the cells after treatment have been

Table 6. TFO−Peptide Conjugates

compd	sequence	ref
95	3′-GGAGGGGGAGGAGGAGGAGG-O-P(O_2)-NH-GGGP-(ADALDDFDLDMLP)$_3$	190
96	3′-GGAGGGGGAGGAGGAGGAGG-O-P(O_2)-NH-GGGP-(ADALDDFDLDMLP)$_2$	190
97[a]	RQIKIWFQNRRMKWKK	193
98[b]	5′-AGGAAGGGGGGGGTGGTGGGGGAGGGGGAG(CH_2)$_3$S-S-3′	193
99[b]	5′-AGTCAGTCAGTCAGTCAGTCAGTCAGTCAG(CH_2)$_3$S-S-3′	193

[a] Antp peptide sequences. [b] TFOs **98** and **99** were synthesized with 5′-rhodamine and 3′-propyl disulfide modifications using rhodamine phosphoramidite and the 3′-thiol modifier C3 S−S CPG, and they were purified by anion-exchange HPLC.

confirmed. The TFO−NLS conjugates cleave the target in a concentration-dependent manner regardless of the presence of the "ballast" oligonucleotide. In contrast, TFO without NLS cleaved the target only in the presence of an excess of the "ballast". The anomalous behavior of TFO with and without NLS conjugation is suggested to be due to the two different cellular uptake pathways followed by TFOs and TFO−NLS conjugates.

Gene expression can be modulated with suitably designed TFOs. The TFOs thus designed bind to the selected sequences of target DNA, thereby inhibiting its transcription. Peptide conjugation to such TFOs is shown to enhance their activity. Similarly, certain TFO−peptide chimera are shown to activate polypurine stretches of a promoter.[189]

Recently, two different TFO−P conjugates, **95** and **96** (Table 6), have been designed and synthesized to activate the target sequences of the promoter (of transcription) shown in Figure 11.[190] In the designed hybrid molecule, the TFO

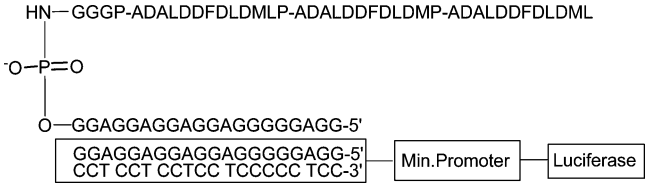


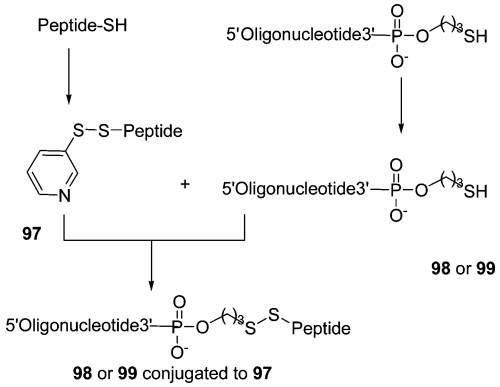
Figure 11. Model of the TFO-P conjugate **95** and its interaction with the target plasmid. The TFO is oriented antiparallel to the purine strand of the target duplex in the plasmid.

is linked to several minimal transcriptional activation domains derived from HSV protein 16 (VP16) through a phosphoroamidate bond as shown in Figure 11. The oligonucleotides of these TFO−P conjugates are designed to form a triple helix specifically to the polypurine stretches of the SIV vpx gene at physiological salt and pH conditions. The TFO−P conjugates (**95** and **96**) were synthesized using the LPFC method, wherein the HOBT esters (1−50 nmol) of the TFO-5′ and TFO-3′ are treated with large excesses of peptide (1.5 μmol) in 0.4 M *N*-methylimidazole (5−30 μL) and incubated at pH 8.0, 0.2 M NaCl, for 12 h at 8 °C. The TFO−P conjugates were then purified (from unreacted oligonucleotides and peptides) by PAGE, followed by elution with 2 M $LiClO_4$, and desalted by acetone precipitation.

Triplex formation of the above synthesized TFO−Ps with target genes was verified by gel-retardation[191] and comigration[192] assays at physiological salt and pH conditions. Similarly, the intracellular stability of the TFO−P was verified by electroporation of the ^{32}P labeled TFO-5′-P_3 and analysis of the cellular extracts by PAGE. No degradation of the product was seen after 24 h of cell growth. Then, the biological activity of the TFO−Ps was assessed using a transient transfection assay. The TFO−Ps containing two transcriptional activation domains (**96**) did not have any influence on the promoter activity. However, the conjugates **95** significantly increased luciferase expression. This effect was dependent on the dose of TFO−P used for triplex formation.

Glazer and co-workers investigated the ability of translocating peptides to enhance the intracellular delivery of TFOs using transport peptides derived from the third helix of the homeodomain of the antennapedia (Antp) peptide (**97**; Table 6).[193] The TFO (**98**) and peptide (Antp) fragments of these conjugates are linked by means of a potentially reversible disulfide bond. The disulfide bond has been chosen mainly because it may be reduced by intracellular enzymes, thereby releasing the oligonucleotide in the cytoplasm and nucleus. The resulting free TFO can now associate with the target gene. However, the reducibility of the disulfide linkage in cells has not been directly established yet. The TFO−Antp conjugates were prepared by the LPFC method as shown in Scheme 17. In this method, the oligonucleotide

Scheme 17



thiol (**97**) is treated with S-activated peptide thiol (**98**) to yield the conjugate (**99**) quantitatively.

The microscopy studies showed that Antp conjugation yields enhanced intracellular uptake of **98**. The TFO−Ps showed a 20-fold increase in mutation frequency as compared to naked TFOs. However, it is reported that **98** itself showed some detectable intracellular uptake. This enhanced cellular uptake of **98** is stipulated to be due to the G-rich nature of **98**, since the oligonucleotide **99** showed lower uptake in the absence of Antp conjugation. Also, the extent of intranuclear accumulation of the **98**−**97** conjugate is reported to be inferior to that of the control TFO (MIX30)−Antp conjugate (**80**−**97**) under the same conditions. From these results, it is believed that the base composition of TFO might influence the efficiency of uptake or the pattern of intracellular distribution following transmembrane delivery.

4.1.3. Antiviral Agents

The therapeutic value of oligonucleotides as antiviral drugs has been studied *in vivo*. In 1998, the FDA approved an antisense oligonucleotide, fomivirsen sodium (Vitravene, Isis

Table 7. Antiviral Oligonucleotides

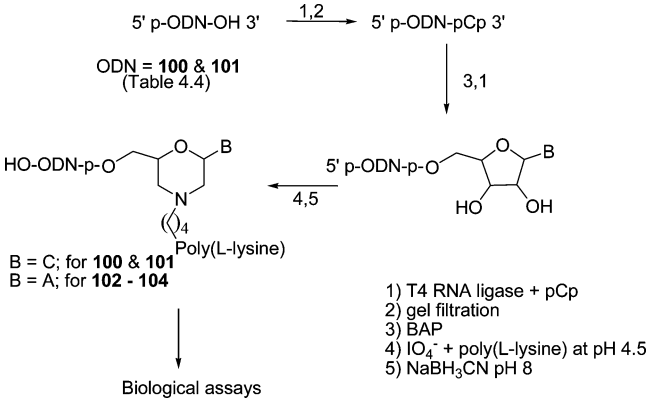
compd	oligonucleotide[a]	viral target	ref
100	5′-d(TTACACGGAGCAA)rC-3′	anti-VSV	198
101	5′-d(TGTCATTAGTTTTAG)rC-3′	anti-VSV	198
102	5′-d(CTAGGATCTACTGGCT)rA-3′	TAT-BRU of HIV-1	199
103	5′-d(CATCGGAGTCTCGATC)rA-3′	TAT-random of HIV-1	199
104	5′-d(TTAGGATCTACTGGAT)rA-3′	TAT-ELI of HIV-1	199

[a] Conjugation of these oligonucleotides to PL was effected as described in Scheme 18.

Pharmaceuticals, Carlsbad, CA), for the treatment of CMV in AIDS patients.[194] This is the first oligonucleotide based drug approved by the FDA, and a few others are under clinical trials.[195] The cellular uptake and, in some case, the antiviral activities of oligonucleotides are shown to be enhanced upon conjugation to the polycationic peptide poly-L-lysine (PL). The cytotoxicity of the PL has been studied well.[196] The naked oligonucleotide methyl phosphonate complementary to the HIV-1 splice donor site did not show any antiviral activity. However, its PL conjugated analogue significantly reduced the viral signal protein.[197] Some of the antiviral oligonucleotides that showed enhanced activity upon conjugation to PL are given in Table 7.

The efficacy of PL conjugation to enhance the anti-VSV activity of the oligonucleotides **100** and **101** was studied. The oligonucleotides are complementary to the initiation region of VSV mRNA.[198] The PL conjugates of these oligonucleotides were prepared in a multistep process as shown in Scheme 18. This involves enzymatic ligation of a

Scheme 18



3′-terminal ribose unit (using T4 DNA ligase) followed by oxidation and reductive coupling to PL to yield the desired conjugates.

The preparation and anti-HIV activity of the oligonucleotides **102**−**104** and their PL conjugates have also been reported.[199] These oligonucleotides were prepared using automated DNA synthesizers, on a riboadenosine derivatized support, using a Biosearch Cyclone automatic DNA synthesizer and purified by RP-HPLC. These oligonucleotides are complementary to different regions of the viral gene (as described in Table 7). The PL conjugates of **102**−**104** were prepared as described for **100** and **101** (i.e., via the four steps in Scheme 18). The PL conjugates of **102**−**104** are shown to possess transient anti-HIV activity in *de novo* infected MT4 cell lines; however, none of the conjugates yields complete protection against viral infection.

Similarly, the antiviral potency of antisense oligodeoxynucleotide (anti TAT) and oligophosphorothioate (S-dC28), before and after conjugation to fusogenic peptide, was evaluated on *de novo* HIV infected CEM-SS lymphocytes in serum-free medium.[200] The conjugates are shown to exhibit better antiviral activities as compared their unconjugated analogues. For instance, the anti-HIV activities of the phosphodiester antisense oligonucleotides are shown to be improved by 5- to 10-fold, after linking them to the peptide by a disulfide or thioether bond, in a one-to-one ratio of peptide and oligonucleotide. The conjugates were observed to be nontoxic at the ED levels (0.1−1 μM), and no sequence specificity was obtained.

Recently, POCs have also been used as adjuvants in therapeutic formulations. A composition containing oligonucleotides conjugated to different peptides through diphosphodiester or diphosphodithioate ester linkages has been developed to treat viral infections and to stimulate the immune systems.[201]

Antisense PNA−HIV Rev peptide conjugates of the type TGCGC-linker-Rev 37−50 were designed and synthesized to be developed as novel RNA-binding molecules.[202] The binding affinities of these conjugates with the Rev responsive element (RRE) RNA were determined by the competition assay using a rhodamine labeled Rev. The results revealed that the PNA or the peptide bound the target RNA (RRE RNA) more efficiently than naked PNA or peptide.[202]

The TAR element in the 5′-long terminal repeat (LTR) of the HIV RNA genome is conversed during the gene mutations. So, TAR has been used as an effective target for oligonucleotide based anti-HIV drug development. Pandey and co-workers have carried out a series of efforts on the synthesis and antiviral activity of several PNA−peptide conjugates.[50,203,204] The PNA **105** (Table 8) is designed to

Table 8. Virucidal and Antiviral Activities of PNA−Peptide Conjugates[a]

compd	PNA[b]−peptide[c] conjugate	IC_{50} for inhibition of HIV-1 replication[d] (nM)	IC_{50} for HIV-1 virucidal activity[d] (nM)
105a	PNA−transportan-27	400	66
105b	PNA−Tat-peptide	720	37
105c	PNA−penetratin	800	28
105d	PNA−transportan-21	1000	34
105e	PNA−transportan-22	1100	72

[a] The antiviral efficacy experiments were carried out in CEM cells infected with the VSV-G pseudotyped S1 strain of HIV-1 by growing them in the presence of increasing concentrations of PNA-MTD peptides. Cells were harvested, lysed, and assayed for luciferase expression after 48 h postinfection. For further details, please see ref 50. [b] PNA = Cys-TCC CAG GCT CAG ATC T- (**105**). [c] For the sequences of the peptides, please see ref 50. [d] For detailed data analysis, please see ref 50.

bind the TAR element region of HIV-1. It has been shown that the anti-TAR PNA (PNA−TAR) upon conjugation with a membrane-permeating peptide (MPP) vector (transportan) retained its affinity for TAR *in vitro* similar to the unconjugated PNA.[204] Examination of the functional efficacy of the PNA−TAR−transportan conjugate in cell culture using luciferase reporter gene constructs showed significant inhibi-

Table 9. Antiviral PMO−Petptide Conjugates

compd	sequence	viral target	ref
106	Pep1[a]-5′-TTC TTT CCA CTA GGC GAA CAA-3′	near 5′-end of genomic RNA of IHNV	205
107	Pep2[b]-5′-GTT CGT TTA GAG AAC AGA TC-3′	by base pairing to specific sequences in the SARS-CoV 53−72	206
108	Pep2[b]-5′-TAA AGT TCG TTT AGA GAA CAG-3′	by base pairing to specific sequences in the SARS-CoV 56−76	206
109	Pep1[a]-5′-GCT CAC ACA GGC GAA CTA CTC-3′	5′-terminal 20 nucleotides of WNV	207
110	Pep3[c]-5′-CCA GGT GTC AAT ATG CTG TTT T-3′	3′-terminal element of WNV	207

[a] Pep1 = R_9F_2C-linker-. [b] Pep2 = R_9F_2C-linker- or R_5F_2 R_4-linker-. [c] Pep3 = CH_3CONH-(R-Ahx-R)$_4$-Ahx-βAla-linker-.

tion of TAT mediated trans-activation of HIV-1 LTR in chronically HIV-1 infected H9 cells. Thus, the PNA−TAR−transportan conjugate can be a potential anti-HIV agent.

The PNA and peptide fragments of the conjugates are linked by means of a disulfide linkage. They have been prepared by following route C of Scheme 13. They have also studied the kinetics of cellular uptake of the CPP conjugates of the PNA **105**.[203] It is shown that the cellular uptake is rapid and that the mechanism of the uptake neither is receptor mediated nor occurs via endocytosis. This has been concluded based on the observations that the cellular uptake characteristics remain unaltered upon changing the temperature and on addition of phenylarsine oxide, which alters the membrane receptor proteins and, thus, the kinetics of cellular uptake. Also, it is noteworthy that the authors have observed a potent virucidal activity, as the HIV-1 viron pretreated with the conjugate became noninfectious.[50]

Similarly, they have also studied the anti-HIV-1 activity of the PNA **105** upon conjugation to five different MTD peptides, namely, penetratin, Tat peptide, transportan-27, and its truncated derivatives: transportan-21 and transportan-22.[50] These MTD peptides are attached to the N-terminal cysteine of the PNA by means of a disulfide linkage, obtained by following route C of Scheme 13. The cellular uptake efficiency of the conjugates was studied by FACS analysis. The penetratin, transportan-21, and Tat peptide conjugates of the PNA (**105**) are shown to be very effective as anti-HIV virucidal agents with IC_{50} values in the range of 28−37 nM (Table 8), while the IC_{50} for inhibition of HIV-1 replication was lowest with transportan-27 conjugated to **105** (0.4 μM), followed by the PNA−Tat conjugate. From these results, the penetratin conjugate **105c** was observed to be highly virucidal, showing the dose median value of 28.0 nM, closely followed by PNA−transportan-21 (**105d**), with a value of 34.0 nM. Another notable observation is that the virucidal activity of **105d** and its cellular uptake are not related to each other. Similarly, the easy reducibility of the disulfide bond can account for the enhanced antiviral activity of the conjugates. Thus, the anti-HIV-1 PNA conjugates for MTD peptides are shown to be not only inhibitors of HIV-1 replication *in vitro* but also potent virucidal agents that render HIV-1 virons noninfectious upon exposure to the conjugates.

As mentioned earlier in section 1.3, PMOs are a class of uncharged single-stranded DNA analogues modified such that each subunit includes a phosphorodiamidate linkage and a morpholine ring. The filoviruses, marburg virus and ebola virus (EBOV), are all negative-strand RNA viruses that cause up to 90% lethality in human outbreaks. There is currently no commercially available vaccine or efficacious therapeutic for filovirus. It has recently been shown that a 22-mer PMO conjugated to arginine rich peptides, wherein the PMO is designed to target the translation start site region of EBOV-VP35 positive-sense RNA, showed sequence-specific, time- and dose-dependent inhibition of EBOV amplification in cell culture.[40] The same oligomer provided complete protection to mice when administered before or after lethal infection of EBOV. Delivery of PMO peptide conjugates targeted against infectious hematopoietic necrosis virus (IHNV) in fish cells *in vitro* and tissues *in vivo* has been examined. The uptake was evaluated by fluorescence microscopy and flow cytometry after treating cultured cells or live rainbow trout with 3′ fluorescein tagged PMO.[205]

The PMO−peptide conjugates of type **106** containing the peptide R_9F_2C attached through a linker at the 5′-end of the PMO showed markedly enhanced cellular uptake in culture by 8- to 20-fold as compared to unconjugated PMOs, when determined by flow cytometry. Enhanced uptake of PMO−peptide conjugates was also observed in tissues of fish treated by immersion.[205] The conjugate **106** showed no inhibition at 0.2, 1, or 2 μm concentrations, moderate inhibition at 10 μm, and marked inhibition (97%) of IHNV infection at 20 μm. Also, at this concentration (20 μm), **106** was shown to be nontoxic to mock-infected cells, as assessed visually. These data demonstrate that replication of a negative-stranded nonsegmented RNA virus can be inhibited by antisense compounds that target positive polarity viral RNA or by a compound that targets negative polarity viral RNA.[205]

The antiviral efficacy of the PMO peptide conjugates against the recently emerged severe acute respiratory syndrome coronavirus (SARS-CoV) has been studied. The SARS-CoV is a potent pathogen of humans and is capable of creating a global epidemic. The peptide conjugates of antisense PMOs were designed to bind the Tor2 strain of the SARS-CoV genome.[206] The conjugates were tested for their capacity to inhibit production of infectious virus as well as to probe the function of conserved viral RNA motifs and secondary structures. Several virus-targeted peptide PMO conjugates and random-sequence control conjugates showed low inhibitory activity against SARS coronavirus. Certain other virus-targeted P−PMOs reduced virus-induced cytopathology and cell-to-cell spread as a consequence of decreasing viral amplification. The PMO peptide conjugates **107** and **108**, targeting the viral transcription-regulatory sequence (TRS) region in the 5′ untranslated region, were the most effective inhibitors among the conjugates studied (Table 9), targeting different regions of the viral RNA.[206] These results suggest that the PMOs and their peptide conjugates have powerful therapeutic and investigative potential toward coronavirus infection. Different PMOs with sequences complementary to RNA elements located in the 5′- and 3′-termini of the West Nile (WN) virus genome were designed to bind important cis-acting elements and potentially to inhibit WN infection.[207] The arginine rich peptide Pep2 was conjugated to each PMO for efficient cellular delivery. These PMO−Pep2 conjugates exhibited various degrees of antiviral activity upon incubation with a WN virus containing cell lines. Among them, PMOs targeting the 5′-terminal 20 nucleotides (**109**) or targeting the 3′-terminal element involved in a potential genome cyclizing interaction (**110**) exhibited the greatest potency. When cells infected with

Table 10. DNA Vaccine−Peptide Conjugates[215]

conjugate	linkage	CpG-ODN structure[a]	peptide[b]
111	thioester	phosphodiester (**117**)	PADRE:19V (**119**)
112	thioester	phosphorothioate (**118**)	PADRE:19V (**119**)
113	hydrazone	phosphorothioate (**120**)	PADRE:19V (**121**)
114	hydrazone	phosphorothioate (**120**)	PADRE:19V (**122**)
115	hydrazone	phosphorothioate (**120**)	PADRE:19V:N:S9L (**123**)
116	hydrazone	phosphorothioate (**120**)	AAA:19V (**124**)

[a] CpG-ODN: 5′-TCCAT*GACGTT*CCT*GACGTT*-3′.

[b]

peptide	sequence
PADRE:19V	AK X[c] VAAWTLKAAAILKEPVHGV
PADRE:S9L	AK X[c] VAAWTLKAAASLYNTVATL
PADRE:19V(N):S9L	AK X[c] VAAWTLKAAAILKEPVHGV-NSLYNTVATL
19V	ILKEPVHGV

[c] X = cyclohexyl-alanine.

an epidemic strain of WN virus were treated with **109** or **110**, virus titers were reduced by approximately 5 to 6 logs at a 5 μM concentration without apparent cytotoxicity. The conjugate **110** also inhibited mosquito-borne flaviviruses other than WN virus. It is reported that the conjugates **109** and **110** suppressed viral infection through two distinct mechanisms. The conjugate **109** inhibited viral translation, whereas **110** did not significantly affect viral translation but suppressed RNA replication.[207]

4.1.4. Enhanced Activity of DNA Vaccines

Genetic immunization is a novel form of vaccination in which transgenes or immuno stimulatory sequences (ISS) of DNA are delivered into the host to produce the foreign protein within host cells. These transgenes are called DNA vaccines.[208] There have been different strategies to enhance the efficacy of such DNA vaccines. One such method of chemist's concern is chemical conjugation of these ISS DNAs with protein[209] and peptides. Albumin[210] and 2-microglobulin[211] conjugates of DNA vaccines have been studied for their immunogenic activity. Such protein conjugates (of DNA) are reported to show better Th1 and CTL responses.[209,212]

Combinations of synthetic ODNs containing unmethylated CpG (cytosine phosphate guanosine) structural motifs and with protein, peptide, or complex antigen mixtures have been studied for their immunogenic stimulatory activities.[213] It is shown that substitution of a natural phosphodiester with a (nuclease resistant) phosphorothioate backbone improved the *in vitro* stability of synthetic unmethylated CpG containing DNAs, and such modification also enhanced its immunogenic properties.[214] Functional conjugates of CpG and its modified analogues with minimal epitope fusion peptides have been studied extensively for the first time.[215] The CpG containing oligonucleotides, peptide sequences, and chemical functionality for conjugates **111**−**116** are given in Table 10. The conjugates were synthesized using two different strategies, namely, the maleimide−thiol and hydrazone formation protocols (Scheme 19) in the liquid phase. The maleimide terminated peptide (**119**) is coupled to ODN thiols, **117** or **118**, as shown in Scheme 19. In the second method, the peptide hydrazines (**121**−**124**) are treated with ODNs containing benzaldehyde (**120**) as shown in Scheme 19.

The immune recognition of DNA−peptide conjugates and complexes of the same was carried out in a human HLA-A2 murine model. A detailed study on the biological characteristics of these conjugates, such as peptide mediated cytotoxicity IFN-γ release and protection against viral infection, was performed. The immunogenicity of the DNA−peptide covalent conjugates was found to be superior in sensitivity to that of noncovalently linked mixtures of the same functional molecules. All these conjugates were found to have robust immunologic activity. For example, in some cases, the conjugate was 10-fold more sensitive than that corresponding to the equivalent mixture of CpG-DNA and peptide.

In yet another report, the NLS peptide conjugates of DNA vaccines are shown to possess superior immunogenic characteristics to those of unconjugated DNA.[216] Here, the authors prepared the hairpin ODNs **125** and **126** (Table 11) and used

Table 11. Sequences of Hairpin ODNs (100, 101) and NLS Peptides (102)[216]

compd	sequence
125	5′-PH-d(GGGAGTCCAGTTTTCTGGAC)-3′
126	5′-PH-d(AGGGGTCCAGTTTTCTGGAC)-3′
127	PKKKRKVEDPYC

them for indirect conjugation of the NLS peptides to the immunogenic plasmid construct in a method similar to that described in section 2.3. This conjugation involves a sequence of biochemical and chemical methods as shown in Figure 12. The *in vitro* transient infection assays were

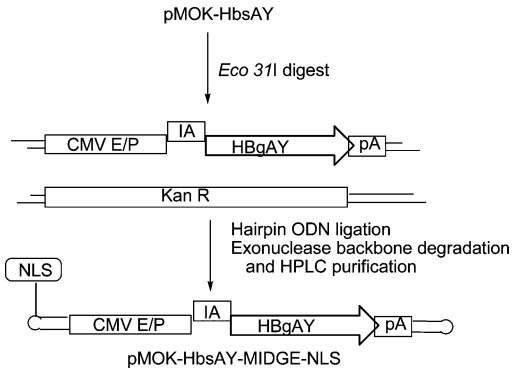
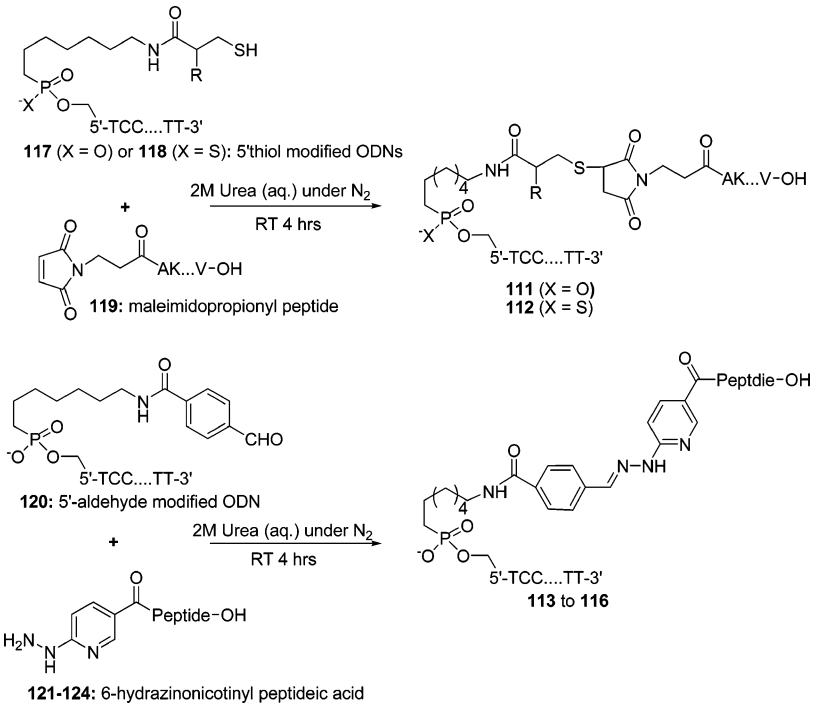


Figure 12. Preparation of the HbsAg-encoding pMOK-HbsAY-MIDGE construct and the pMOK-HbsAY-MIDGE-NLS construct, starting from the pMOK-HbsAY construct.

carried out on the plasmid pMOK-HbsAY, its minimal expression construct pMOK-HbsAY-MIDGE, and its NLS conjugate pMOK-HbsAY-MIDGE-NLS.

The pMOK-HbsAY plasmid was first digested with *Eco 31*I, and either end of the resulting construct was ligated to the hairpin ODNs (**125** and **126**) by T4 DNA ligase with a 10-fold molar excess of the 5′-phosphorylated hairpin ODNs. The mixture was concentrated and treated with *Eco31*I and T7 DNA polymerase in the absence of deoxyribonucleotides. The DNA was purified by anion exchange column chromatography. Then, the NLS peptide (**127**) was coupled to one of the hairpin ODNs in two steps. First, the amino modified (amino-T in the hairpin) oligonucleotide was activated with sulfo-MBS in PBS at room temperature. The activated ODN was isolated and purified with ethanol and buffer solutions. It was treated with peptide at room temperature for about 1 h. The resulting NLS-coupled ODN was purified by HPLC. The biological studies clearly showed that *in vitro* HbsAg expression from the MIDGE-NLS construct is significantly higher than that for unconjugated pMOK-HbsAY-MIDGE. Also, single injection of pMOK-HbsAY-MIDGE-NLS in-

Scheme 19



duced strikingly higher HbsAg-specific serum antibody titers than that of pMOK-HbsAY-MIDGE.

4.1.5. SiRNA–Peptide Conjugates for Gene Silencing

RNA interference (RNAi) is found to be a powerful biological process for specific silencing of gene expression in diversified eukaryotic cells.[217] In this process, a small synthetic RNA fragment (of about 21−23 nucleotides) blocks the expression of target mRNA by binding with its complementary sequences of target mRNA. These RNA fragments are called siRNAs. RNAi has tremendous potential for functional genomics, drug discovery through *in vivo* target validation, and development of novel gene-specific medicines. In the recent past, the RNAs were subjected to various chemical modifications,[218] so as to improve stability, potency, and *in vivo* cellular delivery and to study cellular functions. However, similar to the case of DNAs, the therapeutic value of siRNAs is hindered by poor cellular uptake, limited stability in blood, and nonspecific immune stimulation. To address these problems, different strategies, including linking the siRNAs to ligand-specific, sterically stabilized nanoparticles, have been actively studied.

Similar to its DNA counterparts, RNAs are also shown to exhibit enhanced cellular uptake upon conjugation to peptides. Recently, Scaria and co-workers have shown that functional siRNA can be delivered into cells by using RGD peptide derived nanoparticles (Figure 13a).[219] These self-assembled nanoparticles were constructed with PEG-PEI conjugated to an RGD peptide (Figure 13b). The RGD peptide ligand Arg-Gly-Asp is attached at the distal end of the PEG, to target the tumor cells. The nanoparticles of different size form complexes, called nanoplexes, with the siRNAs to be delivered.

The ability of this nanoplex containing siRNA to silence the expression of VEGF-R2 in tumor cells has been studied. The human cell lines (HUVEC and N2A) and mice models have been used for the experiments. The nanoplexes were administrated into tumor-bearing mice intravenously. From the results, it is shown that the tumor cell expression was inhibited effectively by the siRNAs. Thus, an RGD peptide−PEG-PEI conjugate (**128**) has been used successfully to deliver siRNAs and, thus, to inhibit tumor angiogenesis.

The cellular delivery and RNAi activity of siRNAs upon conjugation to Tat peptide (Tat 47−57) and Tat derived oligocarbamate has been studied.[220] A series of covalent conjugates of Tat and Tat derived oligocarbamates with the siRNAs **129**−**134** (Table 12) were prepared using the maleimido-cysteine protocol in the liquid phase. A modified single strand siRNA containing an amino group linked through a three-carbon linker at the 3′-terminus is annealed with its complementary sense strand (Cy3 labeled EGFP or CDK9 sequences). The resulting duplex siRNAs are then attached to the heterobifunctional linker, sulfosuccinimidyl-4-(*p*-maleimidophenyl)butyrate, in PBS reaction buffer. Then, the cysteine containing Tat (47−57) peptide or oligocarbomate is incubated with the maleimido ds-siRNAs for covalent conjugation.

The conjugates were then purified by PAGE, in which the conjugates were identified by their retarded mobility as compared to the case of unmodified duplex siRNAs. The oligocarbamate backbone used in this study consists of a chiral ethylene backbone linked through a relatively rigid carbamate group (Figure 14). This modified Tat derived oligocarbamate was previously shown to function in a manner similar to that of wild-type Tat peptide in Tat peptide−RNA binding experiments, and it was shown to be resistant to proteinase K digestion.[221]

The cellular uptake and RNAi activity of these conjugates, as well as other synthetic intermediates, to knockdown EGFP and CDK9 expression were studied in detail. The siRNAs conjugated to Tat (47−57) peptide were shown to exhibit efficient RNAi activity as compared to naked siRNAs.[220] For example, the SS/AS 3′-Tat (47−57) EGFP siRNA (**131**; Table 12) showed ∼70% RNAi activity (at a concentration of 200−300 nM), while the unconjugated SS/AS 3′-N3-EGFP siRNA showed no activity. Similarly, the SS/AS−

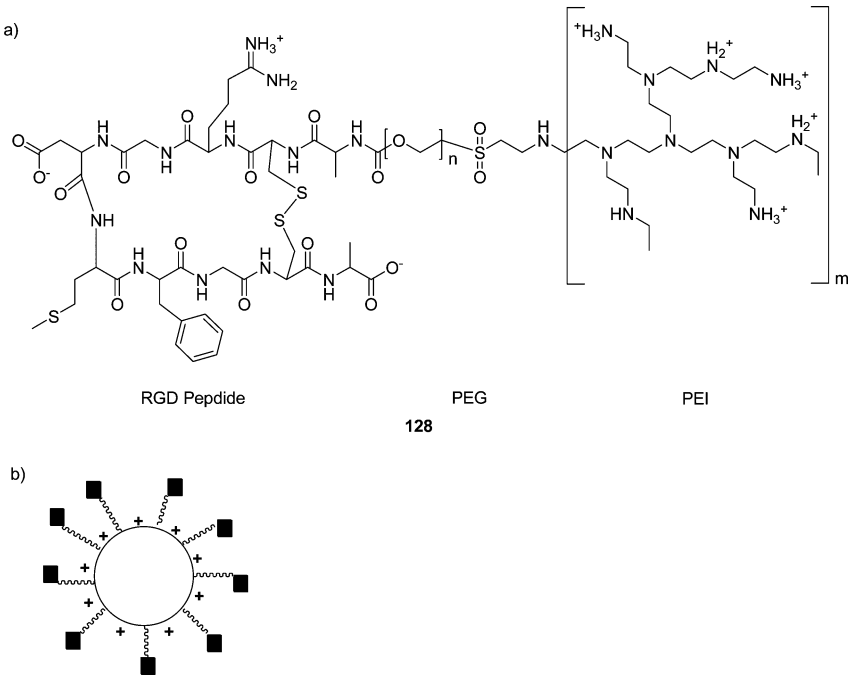


Figure 13. (a) Schematic structure of the RPP polymer containing three different domains, namely, an RGD peptide, PEG, and PEI. (b) Schematic structure of the self-assembled nanoplex, formed by electrostatic interactions between negatively charged siRNA and cationic polymer.

Table 12. SiRNA−Peptide Conjugates[220]

Compound	EGFP-siRNA conjugates	Sequences [a]
129	Cy_3-SS/AS-3'-TAT_{47-57}	Cy_3-GCAGCACGACUUCUUCAAGdTdT TAT_{47-57}-dTdTCGUCGUGCUGAAGAAGUUC
130	Cy_3-SS/AS-3'-TAT_{47-57} (Carbamate)	Cy_3-GCAGCACGACUUCUUCAAGdTdT TAT_{47-57} (Carbamate)-dTdTCGUCGUGCUGAAGAAGUUC
131	SS/AS-3'-TAT_{47-57}	Cy_3-GCAGCACGACUUCUUCAAGdTdT TAT_{47-57}-dTdTCGUCGUGCUGAAGAAGUUC
	CDK9 siRNA conjugates	
132	SS/AS-3'-N3	CCAAAGCUUCCCCCUAUAAdTdT N3-dTdTGGUUUCGAAGGGGGAUAUU
133	SS/AS-3'-TAT_{47-57}	CCAAAGCUUCCCCCUAUAAdTdT TAT_{47-57}-dTdTGGUUUCGAAGGGGGAUAUU
134	SS/AS-3'-TAT_{47-57} (Carbamate)	CCAAAGCUUCCCCCUAUAAdTdT TAT_{47-57} (Carbamate)-dTdTGGUUUGGAAGGGGGAUAUU

[a] Sense (top section) and antisense (bottom section) strands of siRNA species of EGFP (**129**−**131**) and CDK9 (**132**−**134**). The structures of the 5′-Cy3, N3, and 3′-Tat (47−57) oligocarbamate backbones and the sequence of 3′-Tat (47−57) are given in Figure 14.

3′-Tat conjugate **133** was able to show good RNAi activity (∼84%) at a concentration of 400 nM while the unconjugated or other intermediates failed to show RNAi activity (to inhibit the expression of CDK9). The RNAi activity could be observed when the conjugate **107** (at 150 nM) was transfected with Lipofectmine. This is used as a control experiment. The siRNA−Tat derived oligocarbamate conjugates were also taken up by cells and show RNAi activity.

The functional RNAi and siRNA localization, in almost all the cases, was distinctly perinuclear. This is due to siRNA's ability to interact with RISC to cause RNAi at these perinuclear regions. The siRNA perinuclear localization was distinctly different from that of free Tat (47−57) peptide nuclear localization. This is a clear indication of the silencing activityof Tat (47−57) peptide conjugated siRNAs. The authors have also reported a novel method for siRNA delivery using peptide-conjugated nanoparticles. This is also proved to be a viable method for siRNA delivery.

However, it may be mentioned here that the conjugates containing cationic peptides (Tat) have been separated by simple RP-HPLC and purified by 20% nondenaturing PAGE. Under these conditions, there is a possibility that these conditions are not adequate enough to obtain conjugates in the very pure form. Also, based on recent revelations, the cell fixation methods used herein (100% methanol at −20 °C for 10 min) might lead to artifacts in microscopic images. For example, it was shown that cell fixation with methanol induced the artificial nuclear association of VP22 and histone H1 proteins.[222]

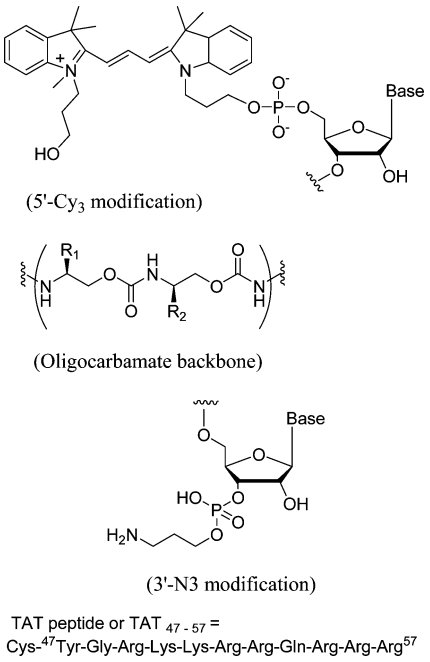


Figure 14. Structures of the 5′-Cy3 and 3′-Tat (47−57) oligocarbamate backbone, and sequence of the 3′-Tat (47−57) peptide of the conjugates **129**−**134**.

The membrane permeant peptides (MPPs), namely, penetratin (**135**) and transportan (**136**), have also been conjugated to improve cellular uptake of siRNAs.[223] The peptides **135** and **136** were linked to siRNA (for e.g. **137**) by means of easily reducible disulfide bonds. The conjugates were prepared by treating thiol terminated siRNAs with cystine terminated peptides as shown in Scheme 20. The single-stranded RNAs were annealed together to form a duplex siRNA, and then, the duplexes were mixed with MPP in equimolar amounts to get the corresponding conjugate. The additional 5′-free thiol group of one of the siRNA strands was attached to a Cy3 fluorescent label for biological studies by fluorescence microscopy. The reaction between the thiol groups on the siRNA and the MPP was catalyzed by the oxidant diamide as shown. The conjugates were purified by HPLC and characterized by MALDI-TOF-MS. The siRNAs of these conjugates are directed to silence firefly luciferase or GFP transgenes. From the biological studies with various cell types, it was shown that these MPP-siRNAs efficiently inhibited transient and stable expression of reporter transgenes in several mammalian cell types in a high proportion of cells. The delivery characteristics of the conjugates were equivalent or even better, in some cases, than that of cationic liposomes, whereas the naked siRNAs (unassisted by penetratin or lipofectamine) could not be taken up by the cells and the luciferase activity remained the same as that in cells transfected only with the transgenes.

Thus, conjugation of MPPs is shown to be a better and relatively inexpensive method for effective delivery of siRNAs to different cell types. In addition, MPPs may complement the use of transfection with cationic liposomes. The gene inhibition using MPP-siRNAs was highly specific. For example, CHO-AA8 cells were more efficiently treated with MPP-siRNAs than with siRNAs transfected using Lipofectamine 2000.

However, here it should be noted that the authors have added the MPP-siRNA and diamide solution directly to the culture media in the biological experiments; this might lead to some erroneous results. This could have been complimented with a control experiment containing only diamide, as done in general.

4.1.6. Increased Stability

In addition to enhancing the cellular uptake, peptide conjugation also plays a vital role in stabilizing the oligonucleotide until its task is accomplished. The peptide conjugation protects oligonucleotides from digestion by the intracellular enzymes present in different cell compartments. Peptide conjugation also enhances the binding ability of the oligonucleotides.

The effect of peptide conjugation on the stability of the duplexes formed by the ODNs has recently been evaluated. A series of POCs, including nucleopeptides, with their complementary ODNs, are as shown in Table 13.[224] The UV melting experiments (T_m values) were used to find out the role of peptide sequences in enhancing the hybridization properties of POCs. Different types of POCs were used for this study. Some of these POCs are self-complementry, while the others are not.

Oligonucleotides and peptides of varying length and compositions were conjugated to obtain the different POCs required for the study. Among the POCs studied, replacement of alanines by positively charged residues (lysine, ornithine, arginine, or histidine) in a peptide covalently linked to an 8-mer oligonucleotide had a greater stabilizing effect than the introduction of hydrophobic residues such as tryp-

Scheme 20

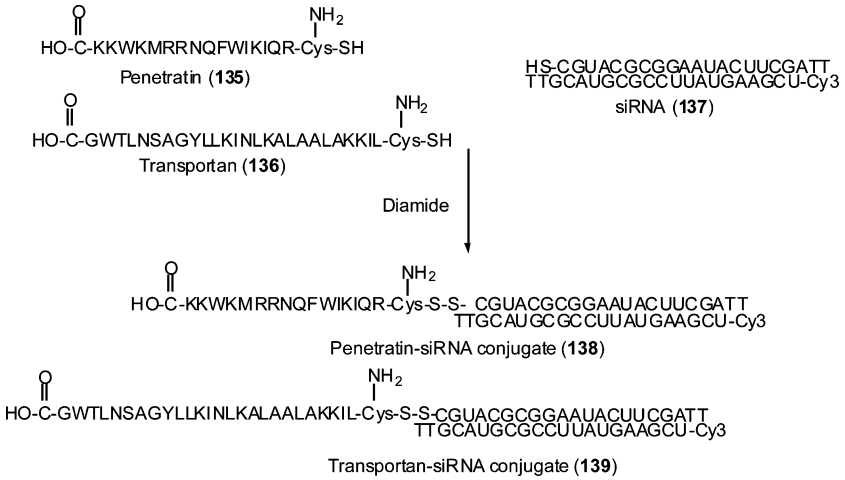


Table 13. Relative Stabilities of Non-self Complementary and Self Complementary POCs[224]

compd	sequence	T_m (°C)	ΔT_m
140	5′-dCATGGCT/3′-dGTACCGA	29.6	std
141	H-linker[a]-5′-dCATGGCG/3′-dGTACCGA	30.0	+0.4
142	HGly-Met-linker[a]-5′-dCATGGCG/3′-dGTACCGA	33.3	+3.7
143	BocHisGly-Met-linker[a]-5′-dCATGGCG/3′-dGTACCGA	31.5	+1.9
144	PhacHis-Gly-Met-linker[a]-5′-dCATGGCG/3′-dGTACCGA	34.1	+4.5
145	AcCys-Gly-Tyr{p3′-(dACTAGT)ProOH}$_2$[S-S][b]	40.0	+32.9

[a] Covalent linkage (linker) between the C-terminal carboxyl of the peptide and the 5′-end of the oligonucletide: $-NH-(CH_2)_6-O-P(O)(O^-)-5'O-$. In entry **142**, the N-terminal of the linker is protonated ($H_3N^+-CH_2^-$). [b] The structure corresponds to the disulfide dimer.

tophan.[224] Most importantly, in all cases, the peptide conjugation showed enhanced stability but with different trends. The stabilizing effect of peptides in conjugates containing self-complementary short duplexes is shown to be much more important. Non-self-complementary sequences showed different behavior upon peptide conjugation. For example, the ΔT_m value of **144** (in Table 13) is much more (+4.5 °C) than that of **141** (+0.4 °C), under similar conditions. Both positively charged and hydrophobic peptides increase the stability of short duplexes. However, the largest stabilizing effect was found in conjugates with self-complementary oligonucleotides, in which both ends are attached to the peptide unit as in **145** (Table 13), which has an intra-peptidyl disulfide linkage. On the other hand, longer duplexes did not show a significant destabilizing effect, when the peptide moiety was either globally uncharged or even negatively charged. The stabilizing effect of a positively charged peptide was relatively small.

Similarly, oligonucleotides appended with a family of cationic peptides containing arginine, lysine, ornithine, and diaminobutyric acid have been prepared. In these conjugates, the oligonucleotides linked through either their α-amino or side chain amino groups have been prepared to study the relative stabilities.[225] Based on their optical melting profiles, the peptides with four positively charged amino acid residues were shown to enhance the triplex-forming capability of the conjugated oligonucleotide. These model studies using different kinds of POCs would help to design better oligonucleotide-based drugs.

4.1.7. POCs in Specific Delivery

Peptides, especially those derived from cell surface receptors, are, by virtue, specific to a particular type of cell. By conjugating these peptides, the oligonucleotides can be directed to those particular cell types. It should be noted that efficient nuclear entry of the exogenous DNA is one of the key factors in the success of gene therapy. In general, oligonucleotides can be directed to the nucleus by attaching NLS peptide sequences. These target specific peptides can be isolated from viral or other pathogenic organisms or can be identified by screening a variety of synthetic peptides.

Shadidi and Sioud have reported a phage-display peptide technology to select peptides.[226] One of the selected peptides was conjugated to an antisense oligonucleotide against the ErbB2 receptor. This POC delivered the oligonucleotide specifically to the breast cancer cells. Similarly, a new strategy to introduce oligonucleotides into the mitochondria of living mammalian cells has been reported.[227] In this method, the N-terminal mitochondrial targeting peptide is covalently coupled to a PNA encoding a portion of the sequence to be introduced into the mitochondrion. This targeting peptide−PNA conjugate is then complexed with oligonucleotide to be internalized. The resulting complexes were imported into the mitochondrial matrix through the outer and inner membrane import channels of isolated mitochondria, but their import into the mitochondria of cultured cells could be done only by using synthetic polycations or membrane permeabilizing toxins, which facilitate cytosolic uptake of the complexes. However, the peptide conjugation has proven to be crucial for cellular uptake. This is confirmed by the observation that the mitochondrial uptake within cells did not occur unless the functional mitochondrial targeting peptide was used.

The NLS peptides derived from the transcription factors, namely, NF-κB, Oct-6, TFIIE-β, TCF-1-α, SV-40, HATF-3, and *C. elegans* SDC-3 (Table 14), have recently been

Table 14. Fluorescent NLS Sequences Used for Cellular Uptake Experiments[228]

compd	name[a]	sequence
146	FL-NF-κB	FL-VQRKRQKLMP-NH_2
147	FL-TFIIE-β	FL-SKKKKTKV-NH_2
148	FL-Oct-6	FL-GRKRKKRT-NH_2
149	FL-TCF1-α	FL-GKKKKRKREKL-NH_2
150	FL-SV-40	FL-PKKKRKV-NH_2
151	FL-HAFT-3	FL-EFKKRRRE-NH_2
152	FL-*C.e.* SDC-3	FL-FKKFRKF-NH_2

[a] FL = fluorescein label.

evaluated for their cellular uptake and subcellular localization characteristics in different types of cancerous cells.[228] Some of these sequences were accumulated in subcellular, acidic compartments, while others were localized in the nucleus. The cellular fluorescence observed from four different cancerous cells, after exposing them to the peptides **146**−**148**, is as shown in Figure 15. The result shows that though all these peptides are cell permeable, they show different propensities for different cells. This observation can be a basis for future efforts to develop cell-specific peptide based delivery systems. Then, the fluorescein labeled 10-mer 5′-GCGTCTAGCT-3′, derived from the Kras oncogene, was covalently linked to the peptide **146** via a disulfide linkage. This conjugate showed enhanced cellular uptake in MCF-7 (breast cancer) cells, but the unconjugated oligonucleotide did not enter the cells.

It is important to note that peptide mediated enhancement of cellular delivery and the specificity of the peptides are independent of each other. Thus, conjugating the target specific sequences does not necessarily lead to enhanced cellular uptake as well. Similarly, the peptide sequences that enhance the cellular uptake do not necessarily direct the oligonucleotides to the target gene. Also, in many cases, the POCs are trapped in the endosomes or cytoplasmic vesicles. In an effort to alleviate this problem, the oligonucleotides directed to a particular target are conjugated to two different peptides or peptides containing two different domains,

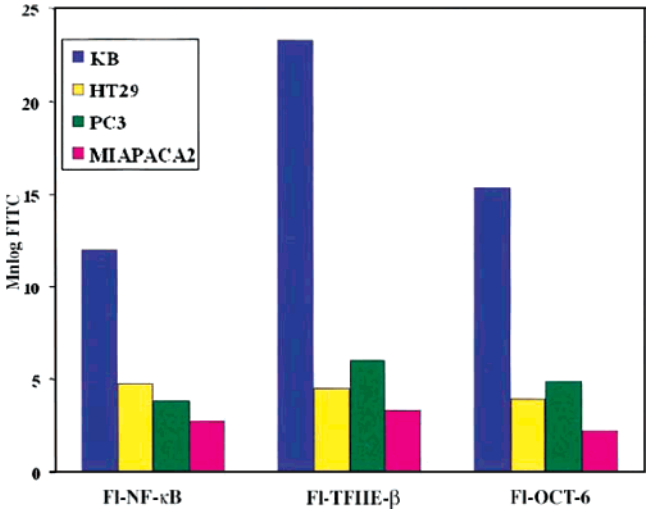


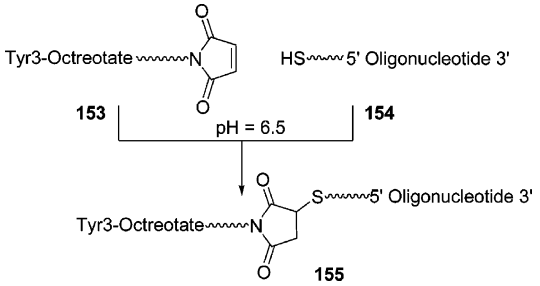
Figure 15. Uptake of the fluorescently labeled NLS sequences FL-NF-$\kappa\beta$, FL-TFIE-β, and FL-Oct-6 (50 μM) with four other cancerous cell types: KB (nasopharengeal), HT29 (colon), MIA-PACA2 (pancreatic), and PC3 (prostate)—after 4 h as measured by flow cytometry. (Reprinted with permission from ref 228. Copyright 2002 Elsevier Sciences.)

namely, one for target specificity and the other to enhance the cellular uptake.

The EGF receptors are overexpressed in many cancer cell types. The feasibility of utilizing this EGF receptor mediated endocytosis to enhance cellular uptake and the specificity of oligonucleotides, in epithelial cancer cells, has been investigated.[229] For this purpose, they prepared a molecular conjugate of EGF and (high molecular weight (~10 000)) poly-L-lysine (PL), joined together by means of a reversible disulfide linkage and SPDP as a cross-linking agent. This EGF−PL conjugate was complexed with 5′-fluorescently labeled 18-mer oligonucleotide phosphodiester (TGT(A)$_4$CGACGGCCAGT). The cellular uptake characteristics of the ON:EFT−PL complex in the presence and absence of two fusogenic peptides, namely, polymyxin B and influenza HA2 peptide (GLCEAIAGFIENGWEG-MIDGGGYC), were studied. Cellular uptake and the intracellular distribution of the oligonucleotide were monitored fluorometrically. Cells treated with the complex exhibited significantly enhanced (up to 12-fold) intracellular fluorescence intensity, over free oligonucleotide-treated controls. The uptake of the complex was shown to occur via the EGF receptor mediated pathway. In the absence of the fusogenic peptides, the complex appeared to be accumulated in endocytic vesicles, but the presence of HA2 peptide and polymyxin B resulted in a more diffused intracellular fluorescence pattern and a corresponding increase in fluorescence intensity.[229] The EGF−PL conjugate may potentially be used as an effective and selective delivery system to enhance uptake of oligonucleotides into cancer cells.

The target (receptor) specific peptides can be identified by screening a library of peptides for the particular receptor. Recently, phage libraries were screened on human breast cancer cell line SKBR3 to identify a suitable peptide sequence for efficient delivery of antisense oligonucleotides to the cancer cells.[230] This peptide sequence, LTVSPWY, exhibited a specific binding to breast cancer cells. The peptide was subsequently linked to an antisense phosphorothioate oligonucleotide against the ErbB2 receptor. In contrast to free antisense oligonucleotide, the peptide−antisense oligonucleotide conjugates inhibited ErbB2 gene expression effectively. Thus, efficient delivery of antisense oligonucleotides can be achieved by coupling them to cancer cell-specific peptides, identified by this method. The peptide sequences, which are analogous to such cell surface receptors, have also been used to enhance the specificity of the antisense oligonucleotides toward that particular cell. For example, Somatostatin receptors (SSTRs) are overexpressed in various tumor cells. Tyr3-octreotate (an analogue of somatostatin) was conjugated to antisense phosphorothioate ODNs (directed against the protooncogene, bcl-2) through a thiol-maleimido link[231] wherein the N-terminal maleimido functionalized peptides (e.g. **153**) were reacted with 5′-thiol derivatized phosphorothioate-ODNs (e.g. **154**) as shown in Scheme 21.

Scheme 21



The conjugates (e.g. **155**) retained their specific binding to SSTRs even in the nanomolar range (IC_{50} values between 1.83 and 2.52 nM). Also, the melting studies with complementary DNA showed that the terminal conjugation of the ODNs did not significantly affect their hybridization affinity.

In general, exogenous DNA can be directed into the nucleus by conjugating it with NLS peptide sequences. Recently, the ODNs **156** and **157** (Table 15) were conjugated to one or two NLS peptide sequences, to give corresponding conjugates to be used as indirect conjugating agents to enhance the nuclear entry of exogenous DNAs.[232] These oligonucleotide−NLS peptide conjugates were then ligated to target DNAs. Three dumbbell-shaped, GFP-encoding DNAs were the targets studied. The peptide was conjugated to the loop-forming regions of the ODNs by cross-linking reactions between the peptide and a modified uracil base with a dioxaoctylamino linker (as described in section 4.4.4). The expression of the NLS-conjugated DNA dumbbells in simian COS-7 cells was studied. From the experiments, it was concluded that conjugation of one or two NLS peptide(s) did not dramatically improve the nuclear entry of DNA. Also, the chemical modification of DNA reduced the transcription efficiency or stability in the nucleus.

Table 15. Oligodeoxynucleotides for Specific Delivery[232]

ODN	sequence[a]
156	d-5′-*GATC*TGGCTCGCCTGTTTTTCAGGCGAGCCA-3′
157	d-5′-*GATC*TGGCTCGCCTGTTXTTCAGGCGAGCCA-3′

[a] X represents the modified uracil derivative conjugated or unconjugated to the NLS peptide. The italicized and underlined sequences represent sticky ends and loop-forming sites, respectively.

4.2. As a Model in Structural and Mechanistic Studies

4.2.1. Mechanisms of Cellular Uptake

Most of the negatively charged naked oligonucleotides are believed to be taken up by fluid-phase or adsorptive endocytosis,[12] but upon conjugation to peptides, their inter-

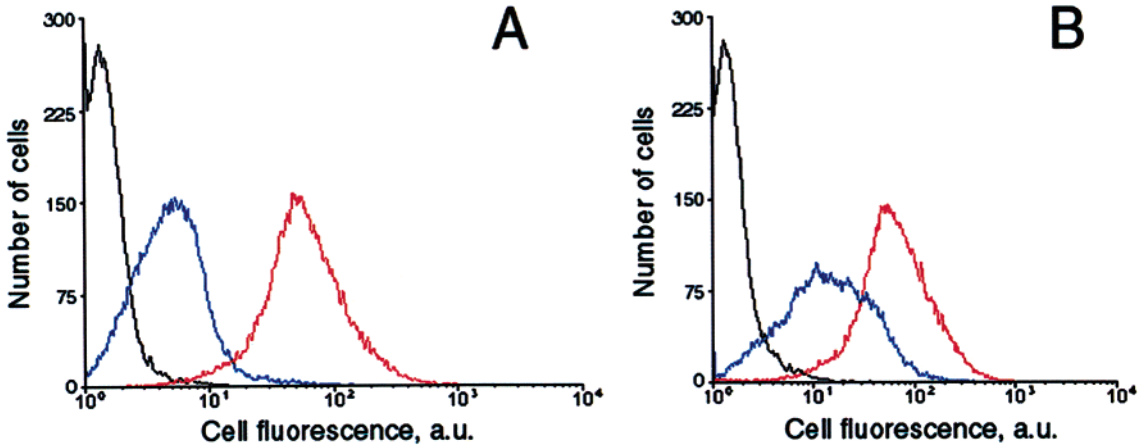


Figure 16. Cellular uptake of Tat-PNA (**158**) is inhibited at 4 °C and by depletion of cellular ATP. HeLa cells were incubated for 15 min in the presence of 1 μM fluorescein tagged Tat-PNA. (A) Cells were incubated at 37 °C (red curve) or at 4 °C (blue curve). (B) Cells were preincubated (blue curve) or not (red curve) with sodium azide and deoxyglucose to deplete cellular ATP. Samples were treated with trypsin before FACS analysis. (Reprinted with permission from ref 235. Copyright 2003 American Society for Biochemistry and Molecular Biology.)

nalization mechanisms differ. Two different mechanisms have been proposed for the cellular uptake by different peptides. One is the energy dependent receptor mediated endocytosis, while the other mechanism is the energy independent membrane translocation, but the exact mechanistic pathway of cellular uptake of POCs still remains a controversy to be solved. Researchers have designed different peptide oligonucleotide model systems exclusively to study the exact cellular uptake pathway. As of now, it is extremely hard to predict the exact pathway followed by a given peptide or protein sequence. The data available so far on the cellular import characteristics of different peptides show some correlation between their structure and mechanism. In an earlier study, the mechanistic pathways followed by Tat proteins and Tat peptides were believed to be different from one another.[233] In contrast to the case of the Tat protein, uptake of Tat peptide does not involve binding to heparin sulfate proteoglycans and endocytosis.

However, recently, the mechanism of uptake of the CPPs such as Tat,[234−236] penetratin, transportan,[234] and $(Arg)_9$[235] has been reevaluated in detail, using different cells, under different conditions. Based on these studies, the authors have pointed out that the cell fixation methods used in earlier reports[233] might have led to redistribution of the peptides before they were observed through microscopy, thereby leading to a wrong conclusion. This is substantiated by the present finding that even mild cell fixation conditions such as using 3.7% (v/v) formaldehyde lead to artifactual nuclear localization of the naked Tat peptide.[235] Now, it is strongly believed that the Tat peptide and penetratin uptake occurs via the endocytosis method whereas, for transportan, the mechanism of uptake still remains highly unclear.

Also, it is shown that, in flow cytometry analysis, it is essential to include the trypsin digestion of the cell membrane adsorbed peptides as an essential step to get correct results. This is because the peptides bind strongly to the cell plasma membrane and remain associated with cells even after repeated washings. The peptide uptake is inhibited at lower temperature (at 4 °C) and at cellular ATP pool depletion. This observation further indicates the involvement of endocytosis. Thus, it is concluded that, in live cells, the peptides show kinetics of uptake that are similar to the kinetics of endocytosis. The authors have also studied the cellular uptake characteristics of an anti HIV-1 TAR PNA−Tat conjugate. The peptide (Tat) and PNA (anti-TAR sequence, CTC CCA GGC TCA (**158**)) fragments of the conjugates were linked by an AEEA linker.[235] This conjugate was prepared by the sequential solid-phase method on Fmoc-PAL-PEG-PS support. The peptide part was assembled first, followed by the PNA part using Fmoc chemistry (for both peptide and PNA assembly). The cellular uptake characteristic of the conjugate was studied by flow cytometry analysis (as shown in Figure 16). This experiment confirms the involvement of the endocytosis pathway in Tat-**158** conjugate as well.

Also, recently, there has been some evidence to indicate that, in primary cells, Tat peptide follows heparan sulfate receptor mediated endocytosis in CHO-K1 cell lines.[236] This is concluded based on the observation that mutant cells that lack the receptor showed significant reduction in cellular uptake of Tat. This is further confirmed from the fact that cells pretreated with heparinase III, an inhibitor of heparan sulfate receptor, inhibited Tat uptake in wild CHO cells. However, there are conflicting results on the involvement of the caveolin-dependent pathway. It is shown that Tat peptides fused to proteins involve the caveolin-dependent pathway,[237,238] but primary cells that lack caveolin-1 also showed efficient uptake of unconjugated Tat peptide.[236] Therein it is also shown that addition of specific inhibitors of clathrin-dependent endocytosis partially inhibited the Tat peptide uptake.

As mentioned earlier, MAPs denote a group of peptides derived from the α-helical amphipathic model peptide **82**.[239,240] MAPs are reported to follow highly complex cellular uptake mechanisms. In some cases, the mechanism involves both energy-dependent and energy-independent processes to a comparable extent. Recently, a detailed study has been carried out to understand the structure−activity relationship of the MAP derived peptides to their uptake characteristics, in mammalian cells.[239] The peptides used are of varying helix-forming propensity, chain length, charge, and amphipathicity as shown in Table 16. The relative cellular uptake of naked phosphorothioate oligonucleotides, the complex, and conjugates of the peptide and the oligonucleotides, in mammalian cells, was observed. A comparison of the data showed that the uptake differences between naked oligonucleotides and their respective peptide complexes or conjugates were very small (generally confined to 1 order of magnitude). This study showed that the structural proper-

Table 16. Sequences and Structural Properties of the Peptides Studied[239]

peptide	sequence	structural properties
159	dansyl-GCKLALKLALKALKAALKLA-NH_2	α-helical, amphipathic
160	dansyl-GCKLGLKLGLKGLKGGLKLG-NH_2	reduced amphipathicity due to strongly impaired helicity
161	dansyl-GCKALKLKAALALLAKLKLA-NH_2	α-helical, nonamphipathic
162	dansyl-GCKGLKLKGGLGLLGKLKLG-NH_2	unsaturated, nonamphipathic
163	dansyl-GCRQIKIWFQNR RMKWKK-NH_2	α-helical, reduced amphipathicity

ties of the peptide components did not have any significant influence upon cellular uptake. This observation is quite contradictory to the widespread belief that membrane permeable peptides enhance the biological activity of oligonucleotides, primarily, by improving membrane translocation of oligonucleotides.

The amphipathic members of the series were enriched within the cell, while the nonamphipathic ones were rapidly washed out of the cell. Extensive translocation into the cell interior was also found for disulfide-bridged peptide and phosphorothioate oligonucleotide conjugates.[240] Unfortunately, the study failed to provide clear information on the relation between the structural characteristics and the cellular uptake of MAPs.

The cellular uptake of naked PNA and the PNA−peptide conjugates (**83** and **84**; Table 4), by rat cardiomyocytes and CHO cells, is as shown in Figure 17.[180] The data show that the conjugates are only partially sensitive to lowered temperature and energy depletion, implying the involvement of nonendocytotic mechanisms in CHO cells. On the other hand, energy-dependent and -independent mechanisms contributed differently to the cellular uptake of the naked PNA and of the conjugate (Figure 17) for cardiomyocytes.[180] This result suggests that distinct modes were functioning in the two cases. Similarly, the conjugates show different sensitivities to the presence and absence of serum. With the information obtained so far, the exact pathways used by different peptides still remain a controversy. Studies to correlate the nature of peptides and their cellular uptake abilities also need to be explored well.

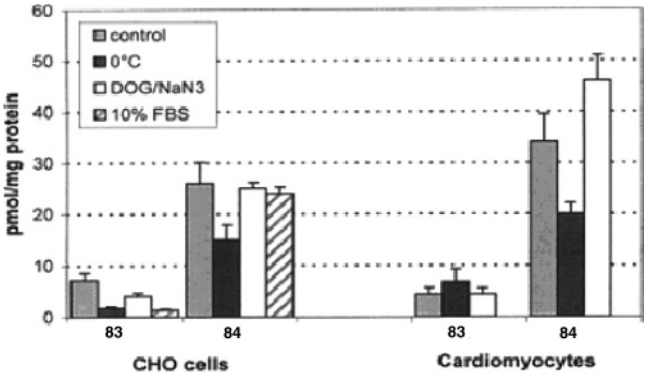


Figure 17. Amount of cell associated PNA determined by CE-LIF in the extracts of CHO cells and cardiomyocytes. Results following exposure to 0.5 μM **83** or 0.2 μM **84** for 30 min at 37 °C without (control) and with energy depletion or in the presence of 10% (v/v) fetal bovine serum (FBS) and at 0 °C. For energy depletion, the cells were incubated in DPBS containing 25 mM 2-deoxyglucose/10 mM sodium azide (DOG/NaN_3) for 60 min at 37 °C and subsequently exposed to the PNA derivative dissolved in the same buffer. To facilitate comparison, the values of **83** were normalized to an exposure at 0.2 μM according to a linear concentration dependence. Each bar represents the mean of three samples ± SEM. (Reprinted with permission from ref 180. Copyright 2004 FEBS, Blackwell Publishing.)

4.2.2. Models To Study Catalytic Activity of Enzymes

Many synthetic POCs also serve as models to understand better the catalytic activity of enzymes, to understand enzyme (protein)−DNA interactions, and to identify the active sites of the enzymes. EcoRII DNA methyltransferase (M.EcoRII) recognizes the 5′...CC*T/AGG...3′ DNA sequence and catalyzes the transfer of the Me group from *S*-adenosyl-L-methionine to the C5 position of the inner cytosine residue (C*).[241] However, the exact region of the M.EcoRII involved in the catalytic reaction is not well-known. To probe the regions of M.EcoRII involved in DNA binding, the covalent conjugates of M.EcoRII−DNA were cleaved by cyanogen bromide. Analyses of the peptide oligonucleotide conjugates obtained from this cleavage experiment led to an understanding about the active site of the enzyme.

The protein−DNA complexes, such as those induced by topoisomerases or the initiation complex in the replication of some viruses, contain nucleopeptides. Hence, nucleopeptides can be used as models for the design of new anticancer or antiviral drugs. In addition, these types of POCs may be useful as nonradioactive labels. The nucleopeptides **164**, **165**, **167**, and **168** were synthesized as models to study tryptophan−DNA interactions.[242]

In these nucleopeptides, the hydroxy group of a homoserine (Hse) side chain and the 3′-end of the oligonucleotide are linked through a phosphodiester bond. The structure and stability of these hybrids were studied by both NMR and CD spectroscopy and by restrained molecular dynamics methods. For example, the three-dimensional solution structure of the complex **164** (formed between the conjugate **165** and its complementary strand, 5′-dCGTAGC) has been

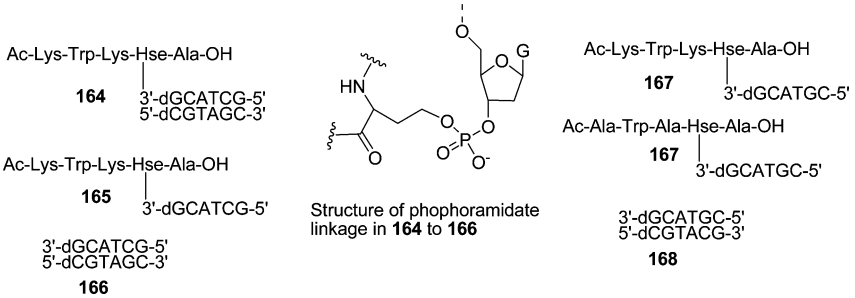
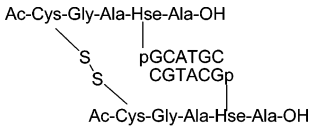


Figure 18. Sequences of the nucleopeptides and DNA duplexes; Hse = homoserine; Ac = acetyl.

determined from a set of 276 experimental NOE distances and 33 dihedral angle constraints. Similar studies on complexes formed by conjugates **167** and **168** with their complementary sequences were performed, and the results were compared with those for duplexes formed by naked ODNs (**166** and **169**). This comparative study helped to understand different factors that contribute to the stability of complexes and other valuable information about interaction.

Conformationally constrained oligonucleotides are interesting model building blocks to be used in construction of a variety of DNA structures, such as hairpins, cruciforms, triplexes, and so on. But, in many cases, these noncanocical DNA structures formed by short oligonucleotide fragments are difficult to study because of their inherent flexibility. However, conjugation of these oligonucleotides to suitable peptides renders the structure stable enough for their complete characterization. For instance, the duplex formed by the nucleopeptide **170** was stable enough to be studied



170

by NMR methods.[243] The structure obtained from the NMR studies was found to be consistent with the proposed 3-D structure of the molecule. This model provided valuable information on the helicity of the oligonucleotide fragment and the basic requirement to enhance the stability of the duplex.

4.3. Artificial Enzymes

A number of molecules of varying size and functionality are known to mimic the natural RNAase. Some of these mimics include oligonucleotides containing monoimidazole[244] constructs and diimidazole or their derivatives.[245] In addition, metal complexes of chelating copper ions and various lanthanides[246,247] also possess RNAase activity. Similarly, a number of synthetic polypeptides have been shown to display RNA cleaving activity, with good efficiency and specificity.[248] The RNAase activity of polypeptides depends on the nature of the amino acids present in them. Short oligopeptides with alternating hydrophobic and basic amino acids displayed negligible RNAase activity, but conjugation of these peptides to ODNs is shown to improve their RNAase activity dramatically but not their DNAase activity. The efficiency of hydrolysis of an artificial enzyme is controlled by the catalytic properties of the active groups, while its specificity is determined by the affinity of compounds for certain RNA sites. The specificity can be tuned by attaching antigenic oligonucleotides that are complementary to the target mRNA.

Mironova and co-workers have synthesized a series of POCs capable of cleaving RNAs in a sequence specific manner.[248−251] The oligonucleotides **171**, **172**, and **173** with different specificities were linked to the core (catalytic) peptide sequence, [Arg-Leu]$_4$-Gly, using the SPFC method to obtain the conjugate shown in Figure 19.[250] The oligonucleotide and peptide fragments of these conjugates (pep−**171**, pep−**172**, and pep−**173** in Figure 19) were connected by *N*-alkylphosphoramidate linkages between the 5′-terminal phosphate of the oligonucleotide and the N-terminal-α-amino

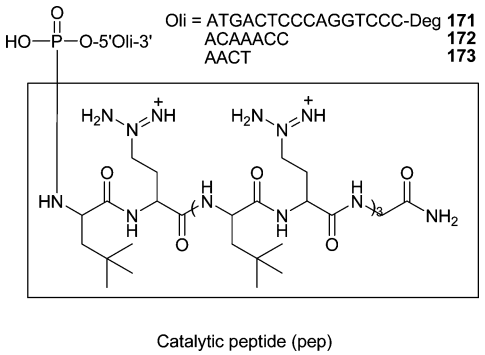


Figure 19. Oligonucleotide−peptide conjugates; pep-**171**, pep-**172**, and pep-**173**, where **171**, **172**, and **173** indicate the respective oligonucleotides and pep corresponds to the peptide [Arg-Lue]$_4$-Gly-NH_2, Deg, diethyleneglycol.

group of the corresponding oligopeptide. The conjugates isolated were purified by RP-HPLC on LiChrosorb RP-18 columns. The authors have carried out a systematic study on the effect of oligonucleotide conjugation upon the activity as well as the specificity of the catalytic peptides.

The conjugate pep−**171** was shown to cleave the *in vitro* transcript of human tRNA$^{Lys}_3$ under physiological conditions.[249] The cleavage was specific at the C56−A57 phosphodiester bond of the RNA target with high efficacy, but the conjugate also cleaved several other bonds in the D loop and in the anticodon hairpin regions. This nonspecific cleavage is believed to be due to the formation of imperfect complexes of the conjugate with partially complementary sequences in the RNA structure. It was found that a 29-mer DNA enzyme conjugated with spermine (using the SPFC method) at its 5′-end showed higher affinity for the target RNA sequence and 40 times higher activity of cleavage than native DNA enzyme. It is also noted that conjugate DNA enzymes showed increased resistance against nuclease digestion.[252] All the conjugates shown in Figure 19 functioned as true catalysts, with a reaction turnover of up to 175 for 24 h. Based on these data obtained on the structural specificity and kinetics of the conjugates, the oligonucleotide is proposed to provide an "active" conformation of the peptide via intramolecular interactions. The peptide residue is responsible for substrate affinity and catalysis.

Similar to almost all the synthetic RNAase known so far, the conjugates pep−**171** and pep−**172** cleaved the phosphodiester bonds in the Pyr-A motif. But the conjugate pep−**173** displayed pronounced G−X specificity. Cleavage at Pyr-A sequences is a typical specificity of synthetic RNases; however, cleavage at G−X motifs is reported for the first time.[251] The exact reason behind the unusual G−X specificity of the pep−**172** conjugate could not be explained fully. The affinity of conjugates to RNA is characterized by a Michaelis constant $K_m = 1.531 \times 10^4$ M. This K_m value is reported to be indicative of some specific complex formation between the RNA substrate and the conjugate during the reaction process. The RNA cleavage is dependent on conjugate concentration.

The peptide conjugates of the ODN T_4 (pep−T_4) are shown to possess varying degrees of RNAase activity depending upon the composition of the peptide sequences.[251] All these peptides contain one or more arginine, leucine, proline, or serine residues. The RNase activities of the conjugates were found to decrease in the following order: T_4-$(LR)_4G$ > T_4-$(LR)_2G$ > T_4-$(LLRR)_2G$ > T_4-$(LR)_2PRLRG$ > S_2R_3-Hmda-T_4 ≥ R_5 ≠ $(LR)_3$. Thus, the conjugates

containing peptides with alternating arginine and leucine residues ((LR)$_4$G-amide) displayed the highest activity. The CD spectral studies were carried out on the free peptide (LR)$_4$G-amide and its conjugate, T$_4$−(LR)$_4$G-amide, in water solution at neutral pH and physiological ionic strength. From the results, it is concluded that the conjugation of ODN induced folding of the peptide similar to the α-helix formation. The highest catalytic activity of the conjugate is explained as due to this α-helix formation of T$_4$−(LR)$_4$G-amide.

It should be noted here that most of the successes in design of RNA cleaving POCs reported so far have come with the use of tRNA substrates. The failure to obtain successful cleavage in POCs targeted to mRNAs makes one think that the cleavage sites in tRNAs are not typical of the cleavage site in mRANs (i.e. are not typical of sequences in mRNA). Further experiments are necessary to understand this better.

Trifunctional conjugates of the type **174** (Figure 20),

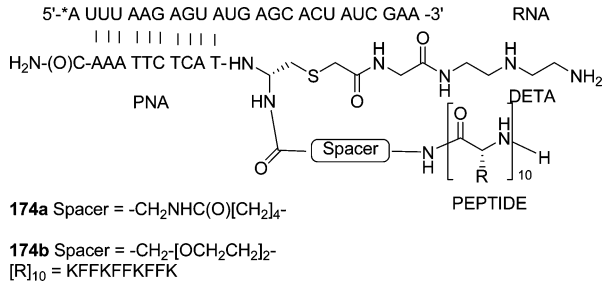


Figure 20. Structure of new trifunctional artificial ribonucleases **174a** and **174b** containing an RNA-recognizing PNA strand, a DETA moiety for RNA cleavage, and a delivery peptide to enhance cellular uptake of PNA.

containing PNA−peptide conjugates, have been synthesized, for the first time, as artificial RNases with potentially improved cellular uptake.[52]

The design of the conjugate is based on the specific RNA-recognizing properties of the PNA, the RNA-cleaving abilities of diethylenetriamine (DETA), and the peptide (KFF)$_3$K for potential uptake of the conjugate into *E. coli*. The decameric PNA derivative of the conjugates **174** is complementary to the start codon region of the mRNA of the essential *E. coli acpP* gene. First, the N-terminal *S-tert*-butylthio protected cysteine residue was assembled on a solid support according to standard PNA synthesis protocols following the Fmoc strategy. Then, the conjugates were prepared in a convergent synthetic route involving native chemical ligation, through a urethane bond. These trifunctional conjugates were evaluated for their RNA-cleaving properties *in vitro* on a 25-mer RNA target and showed efficient degradation of the target RNA at two major cleavage sites. It was also established that the cleavage efficiency strongly depended on the type of spacer connecting the PNA and the peptide.[52] The conjugate **174a** was three times more potent that the conjugate **174b**, though structurally both are very similar except for the linkage. It is perhaps the first report on design and synthesis of PNA−peptide conjugates with RNA-cleaving ability. However, the role of peptide on the cleavage activity of the conjugate could not be established clearly; indeed, the peptide conjugation reduces the activity as compared to those of the corresponding PNA−DETA conjugates.

4.4. Miscellaneous

4.4.1. POCs as Aptamers

Tailor-made aptamers, which are specific for desired molecules among a pool of similar molecules, are of much interest in many chemical and biological applications. A number of such functional RNA aptamers with desired specificity and catalytic activity have been identified.[253,254] Some of these natural RNA−protein complexes have been used as models for design and synthesis of artificial aptamers. For example, recently, an ATP binding aptamer consisting of an RNA subunit and a peptide subunit has been designed by means of a structure-based design approach and a successive *in vitro* selection method.[255] The RNA subunit of this conjugate consists of two functional domains, namely, an ATP binding domain with 20 randomized nucleotides and an adjacent stem region that serves as a binding site for the RNA-binding peptide.

Recognition of peptides and proteins in their live form becomes important, especially in proteomics. Novel aptamers capable of visualizing such protein and supramolecular complexes *in situ* have been designed.[256] The double stranded DNA (dsDNA) conjugated to the prokaryotic DNA-binding proteins LacI and TetR peptide tags have been used in *in situ* imaging studies by means of correlative light and electron microscopy. The dsDNA−peptide conjugates were proven to be capable of multiplex detection of proteins *in situ*. Magnetite core nanoparticles functionalized with aptamers (for rapid affinity extractions) and a C18 functionalized silica nanoparticle (for hydrophobic extractions) have been used for extraction and analysis of peptides using an atmospheric pressure matrix-assisted laser desorption/ionization (AP-MALDI) spectrometer.[257]

4.4.2. POCs in Strand Invasion

Strand invasion is a noninvasive technique to get information about live duplex DNA. In strand invasion, an oligonucleotide or an oligonucleotide mimic binds to its complementary sequence within duplex DNA by Watson−Crick base pairing to form a three-stranded complex. One of the strands in the target duplex is eventually displaced by this strand invasion (Figure 21).

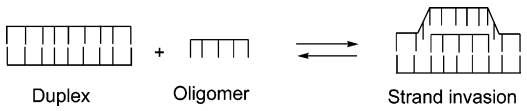


Figure 21. Hybridization of an oligomer to duplex DNA by strand invasion.

Linear PNA,[258] bisPNA clamps,[259,260] bis PNA−DNA conjugates,[260] and bis-PNA-anthraquinone imide conjugates[261] have all been synthesized as potential agents for effective strand invasion. Corey and co-workers have studied the ability of cationic peptide conjugates of oligonucleotides (DNA-CP) and of PNAs to hybridize inverted repeat sequences within supercoiled dsDNA by Watson−Crick base-pairing.[262] The results led to the conclusion that the PNAs and DNA-CPs initiate and maintain strand invasion under more stringent conditions than do unmodified DNA alalogues. Hybridization of DNA-CPs and PNAs to sequences throughout plasmid pUC19 was examined in an effort to elucidate the rules underlying the strand invasion.[263]

An important observation related to the peptide conjugation is that the hybridization of such oligonucleotide ana-

Table 17. Sequences of the Oligonucleotide−Peptide Conjugates Used in Assembly of Nanomaterials[67]

conjugate	sequence[a]
175	5′-TTTTT-3′-hexyl-CONH-AEQKLISEEDLN-CONH-$(CH_2)_5$-OH
176	3′-TCTCCTCCTTC-TTTT-5′-5′-GAAGGAGGAGA-3′-hexyl-CONH-AEQKLI-SEEDLN-CONH-$(CH_2)_5$-OH
177	3′-CGTAACTCGCTACGTCCGTC-$(EG)_6$-bpp-hexyl-CONH-AEQKLISEEDLN-CONH-$(CH_2)_5$-(OH)-$(EG)_6$-CTGCCTGCATCGCTCAATGC-3′

[a] $(EG)_6$, hexaethyleneglycol; bpp, $[-PO_3-O(CH_2)_4-CONH-CH_2]_2-CHOPO_3-]$.

logues, to inverted repeat sequences within normally supercoiled DNA, can be promoted by attachment of a cationic protein or cationic peptides to the oligonucleotide. The association constant K_a for conjugate binding is increased up to 48 000-fold for DNA-CPs as compared to the naked oligonucleotides. The POCs and PNAs were classified into three classes based on their hybridization efficiencies. Hybridization of DNA-CPs and PNAs was stringently dependent on target sequence and was most efficient at sequences within the promoter for β-lactamase or prior to the *E. coli* origin of replication.[263] This finding suggested that the sequences that regulate biological function may also be among the most susceptible to strand invasion.

Nielsen and colleagues have reported that PNAs can bind to accessible DNA within the transcription bubble created by RNA polymerase.[259] To increase the versatility of strand invasion by PNAs, tail clamp-bisPNAs (TC-bisPNA) and TC-bisPNA−peptide conjugates containing a mixed base extension of the Watson−Crick polypyrimidine strand have also been used.[264] It is significant to note that DNA recognition occurs with single-stranded bisPNAs as well as TC-bisPNAs but requires attachment of positively charged amino acids for effective binding. Thus, the TC-bisPNA, upon conjugation to the highly cationic peptide D-$(AAKK)_4$ (containing D-amino acids), showed much higher affinity as compared to the unconjugated TC-bisPNA. For example, the association constant of the TC-bisPNA conjugate TCG ACT CTC CTC CTT-$(AEEA)_3$-TTC CTC CTC T-D-$(AAKK)_4$ for binding the target dsDNA is 35 000 M^{-1} s^{-1} while the unconjugated analogue failed to recognize the target.[264] The PNA and PNA peptide conjugates were prepared by the automated solid-phase method. In bisPNA, the two PNA fragments are separated by a multiple-AEEA linker. The ability of the bisPNA and bisPNA peptide conjugates to bind duplex DNA was monitored by a gel shift assay. This study suggests that TC−PNA-peptide conjugates can be good candidates for further testing as antigene agents.[264] These studies expand the range of sequences within duplex DNA that are accessible to PNAs.

Recently, PNA−peptide chimeras have been used in cooperative strand invasion of supercoiled plasmid DNA, thereby anchoring the biologically active peptides to plasmids in a sequence specific manner. This technology, referred to as "Bioplex technology", has recently been used by Smith and co-workers to attach different peptides to plasmid DNAs.[265,266] The authors have developed a method to quantify the specific binding of PNA using a PNA labeled with a derivative of the fluorophore thiazole orange (TO).[265] Herein it is reported that the linear PNA peptide conjugates bind to a site on the same side of the target DNA, with the highest cooperative effect of two base pairs. Similarly, it has been shown for the first time that in certain cases strand invasion can be facilitated by using LNA together with PNA−peptide conjugates.[266] Herein, LNA oligomers have been used as "openers" to partially overlapping sites on the opposite DNA strand, while the functional peptide conjugates of PNA were used to bind the plasmid DNA. Inclusion of LNA is anticipated to reduce nonspecific interactions and to increase binding kinetics. This procedure allowed hybridization at reduced PNA-to-plasmid ratios (as low as 2:1), but with greater extents of hybridization (80%).

4.4.3. POCs in Nanomaterials

Recently, the POCs **175**−**177** (Table 17), which are capable of forming triplexes and of directed assembly into nanomaterials, have been designed and synthesized.[67] These POCs were prepared by a solid-phase sequential method. The peptide was assembled first using Boc protected amino acid building blocks. Subsequently, the oligonucleotides were assembled using nucleoside phosphoramidite building blocks on the same support. The structures of the POCs were confirmed by mass spectrometry, by gel electrophoresis, and by hybridization with complementary oligonucleotides. The oligonucleotides carrying the c-*myc* peptides were specifically recognized by the anti-c-*myc* monoclonal antibody.

As mentioned earlier, it is shown that functional siRNA can be delivered into cells by using RGD peptide derived nanoparticles.[219] The ability of the nanoplex containing siRNA to silence the expression of VEGF-R2 in tumor cells has been studied using human cell lines (HUVEC and N2A) and mice models. It is shown that the tumor cell expression was inhibited effectively by the siRNAs.

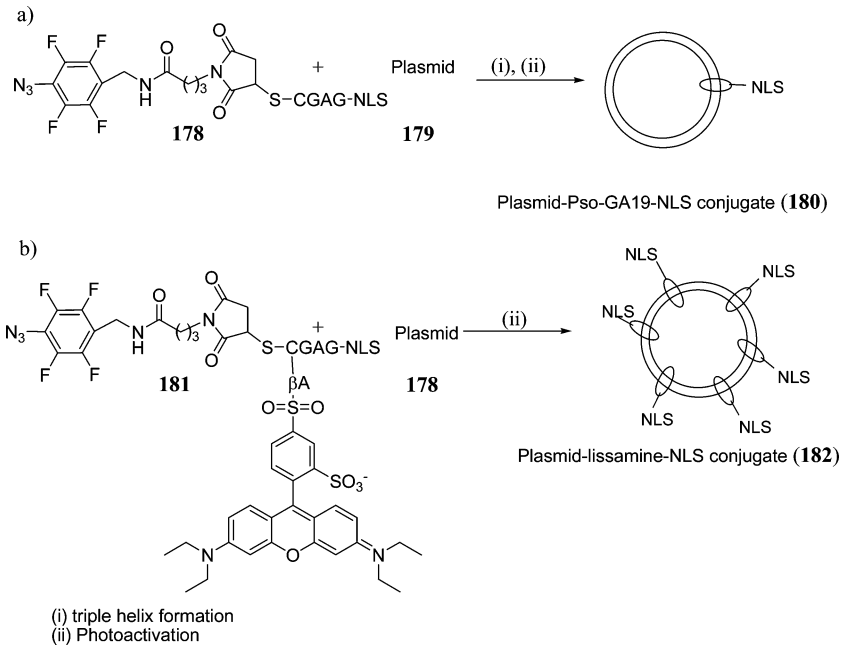
4.4.4. POCs Used in Indirect Conjugation

Direct attachment of proteins to nucleic acids has been a complex task, but it can be achieved by designing suitable peptide oligonucleotide conjugates. The oligonucleotide part binds the complementary site, while the peptide part enhances the cellular uptake of the target nucleic acid.

In general, in the peptide mediated cellular delivery, the antisense oligonucleotide is directly conjugated to the peptide of choice. In some cases, the target DNA, generally the plasmid DNA, is conjugated to the peptide sequence indirectly. This protocol involves a series of chemical methods followed by enzymic methods. In a typical strategy, the chemically linked POC is incubated with the plasmid DNA in the presence of DNA ligase (DNA T4 ligase or DNA T7 ligase), which catalyzes the formation of a chemical linkage between the plasmid DNA and the oligonucleotide fragment of the POC.

For example, two different strategies have been designed and executed for covalent coupling of NLS peptides of SV40 of the large T antigen to plasmid DNA as illustrated in Scheme 22.[267] A TFO−NLS peptide conjugate is used for site-specific labeling of the plasmid DNA with NLS peptides. This is a two step process with site specific triple helix formation of the TFO−NLS peptide conjugate with the plasmid DNA followed by photoactivation as shown in Scheme 22a. The resulting 1:1 complex of plasmid and DNA−NLS peptide conjugate remained fully active in cationic lipid mediated transfection. It has been shown that

Scheme 22



even more than one NLS peptide can be conjugated to the DNA using a similar methodology (Scheme 22b). Even up to 300 fluorescent photocleavable derivatives of NLS peptide molecules could be attached to one plasmid DNA molecule. In this methodology, *p*-azidotetrafluorobenzyllissamine was used as the photocleavable moiety. Both the oligonucleotide-NLS (**180**) and plasmid-lissamine-NLS (**182**) conjugates interacted specifically with the NLS receptor importin-α, but unfortunately, none of the two conjugates, **180** or **182** (in Scheme 22), were detected in the nucleus, after cytoplasmic microinjection.

PNA clamps with the ability to bind irreversibly and specifically to a binding site cloned into the plasmid have also been tried for indirect conjugation of peptides to plasmid DNA.[268] In one such strategy, streptavidin binding sites were introduced into the plasmid DNA by linking a biotin-conjugated PNA clamp into the plasmid DNA. The modified plasmid DNA was shown to form a stable association with oligonucleotide or peptides conjugates of streptavidin. Streptavidin−peptide and streptavidin−oligonucleotide conjugates were prepared by using the maleimido-thiol protocol wherein the peptides and oligonucleotides containing free thiol are treated with streptavidin maleimide. These streptavidin conjugates were bound specifically to the biotin−PNA labeled plasmid. In addition, it has also been shown that a maleimide-conjugated PNA clamp can be used for conjugating thiolated peptides and oligonucleotides directly to the maleimide−PNA−DNA hybrid. So, by using such a methodology, various functional groups can be introduced onto plasmid DNA without disturbing its transcriptional activity. Proteins and oligonucleotides can also be conjugated by this technique.[268]

The ligand induced triplex formation has been used as a primary step to attach padlock oligonucleotides and other targeting moieties to supercoiled plasmid DNA at specific sequences.[269] First, a hairpin forming oligonucleotide is conjugated to the peptide using the maleimido-thiol protocol as shown in Scheme 23. The TFO forms a sequence specific triplex with the target plasmid DNA. The TFO is designed in such a way that it can form a triple helix with the oligopurine·oligopyrimidine sequences, in the phage f1 replication origin of the plasmid DNA. Then, the extremities of the TFO hybridize to each other, leaving a dangling single-stranded sequence, which is then ligated to a hairpin oligonucleotide (**183**) using T4 DNA ligase as shown in Scheme 24.

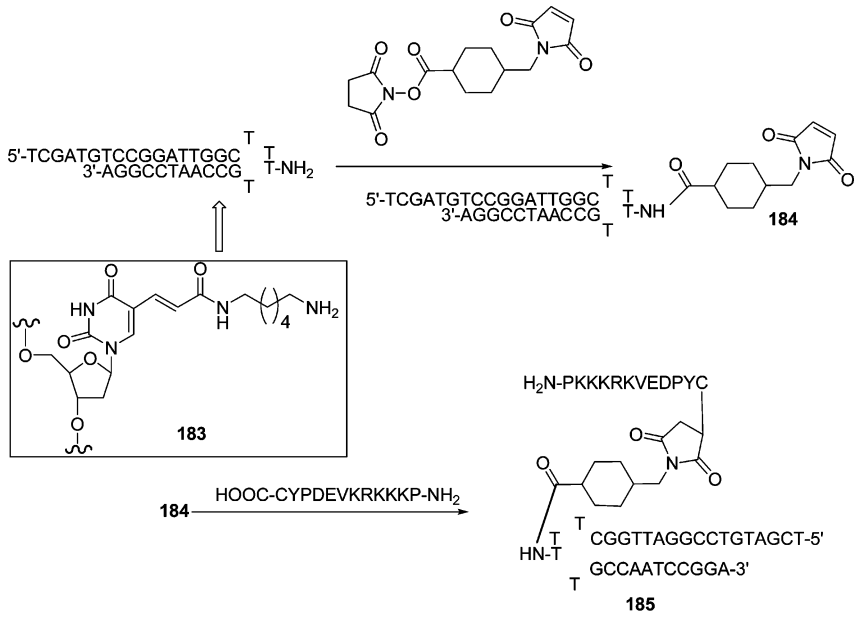
In the above example, an NLS peptide was attached to a luciferase-expressing plasmid DNA using this strategy. Despite the presence of the padlock oligonucleotide, the reporter gene was efficiently expressed after transfection of the plasmid in HeLa or T24 cells, using either cationic lipids or cationic polymers as transfecting agents. However, no increase in gene expression could be observed as a result of peptide attachment. Nonetheless, it is worth mentioning that this technique is reported to be useful for attaching peptides and any other targeting moieties to the plasmid DNAs.

A bifunctional PNA conjugate consisting of a PNA and a NLS peptide has been designed and synthesized for indirect conjugation of the target DNA.[270] The PNA−NLS conjugate was complementary to the target oligonucleotide (containing a fluorophore moiety). The complex of PNA−NLS conjugate and oligonucleotide (bioplex) was transfected to different mouse organs such as liver *in vivo*. The effects of bifunctional peptides in intradermal/subcutaneous transfection and in intramuscular injections were investigated. From these experiments, it was shown that the bioplexes themselves can enter cells efficiently, they may also disperse more easily once inside the cells, and they can eventually be localized in the nucleus. The NLS portion of the bioplex plays a dual role. First, it changes the physical properties of the hybridized oligonucleotide so as to increase its intracellular uptake, and second, it provides a specific signal for nuclear translocation after the oligonucleotide is internalized. The NLS mediated nuclear translocation of nucleic acids was observed in all cells tested, but with much variation with cell types.

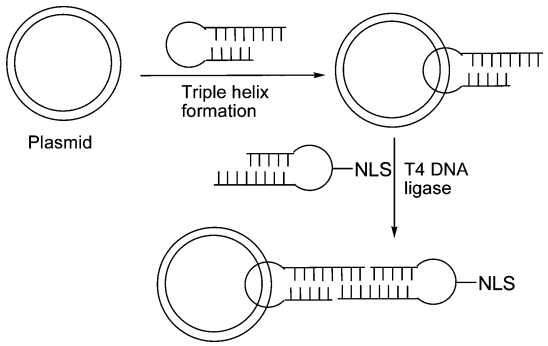
4.5. Metal Complexes of POCs and Their Applications

The metal complexes of synthetic POCs have been used as model systems to study the catalytic activity of enzymes.

Scheme 23



Scheme 24



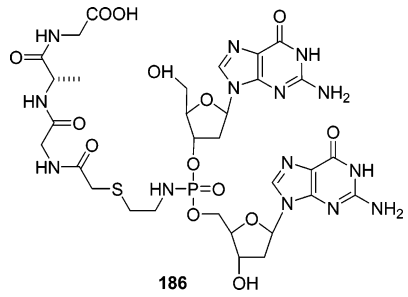
A series of ruthenium(II) bipyridine conjugated peptide complexes have been synthesized as models to mimic the active site of thiamin-dependent enzymes.[271] The interaction of these metal coordination compounds with oligonucleotides has been studied well. The crystal structure, stability, and other physiochemical properties of these complexes are also described therein.

The interaction of bivalent metal ions such as cobalt(II), copper(II), and zinc(II) with purine nucleotides (5′-GMP and 5′-IMP) and dipeptides (DL-alanyl-alanine and DL-alanyl-phenylalanine) has also been studied. These metal ions interacted with the nucleotides or peptides in binary as well as ternary mode.[272] The thermodynamic parameters of these complexes, in different stoichiometric ratios and at different pH, have been studied to identify the most stable complex formed.

4.5.1. Models To Study the Carcinogenicity or Anticancer Activities of Metals

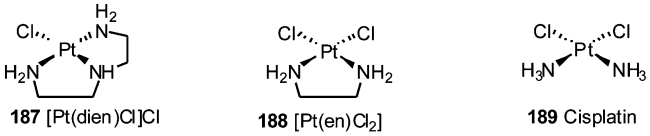
The metal complexes of POCs are also used to study the carcinogenicity of the metals, as well as the anti-neoplasitic activity of anticancer drugs. Chromium is a carcinogenic metal, which may produce DNA damage by two different pathways depending upon the oxidation state of the metal.[273] High valent chromium intermediates lead to oxidative DNA damage, forming free radicals. The low valent chromium (III) forms adduct with the nucleotides. It has been shown that exposure of animals and humans to Cr(IV) complexes has produced amino acid−Cr(III)−DNA adducts, and Cr-(III)-containing DNA−protein cross-links (Cr-DPC). These Cr-containing DNA lesions are mutagenic and are likely to be involved in Cr-induced cancers because they are more slowly repaired than strand breaks.[273]

Stearns and co-workers have designed and synthesized the first dinucleotide peptide conjugate to form a coordination complex with chromium (III), **186**. This complex was used

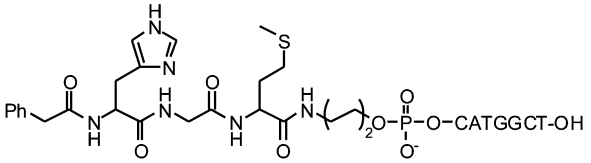


as a model to study the nature of DNA lesions formed in chromium-induced cancers.[273] Conjugate **186** was prepared by coupling the peptide derivative $ClCH_2CO$-Ser-Gly-OH with the dinucleotide dGp($NHCH_2CH_2SH$)dG through a thioether moiety in solution phase. The conjugate obtained was purified by HPLC and characterized by mass spectrometry. They also reported a modified procedure for synthesis of C-terminal chloro acetyl peptide derivatives and dinucleotides (as precursors) on gram scale.

Similarly, the anticancer activity of the platinum (II) complexes **187**, **188**, and **189** has been studied using model peptide oligonucleotide hybrids of type **190**. The hybrid **190** possesses sulfur and nitrogen ligands, which play an important role in coordination with platinum.[274] Thus, the Phac-Met-linker-p5′dG, Phac-His-linker-p5′dG, Phac-His-Met-linker-p5′dG, and Phac-His-Gly-Met-linker-p5′dCATGGCT moieties, which all contain either S or N sites, have been prepared and used in this study. The progress of the reactions of complexes **187**−**189** on hybrid **190** was studied using chromatographic and spectroscopic methods. The coordination sites of platinum on different hybrids were analyzed by characterizing the products obtained from enzymatic and



187 [Pt(dien)Cl]Cl 188 [Pt(en)Cl$_2$] 189 Cisplatin



190

chemical reactions on these complexes on hybrid **190**. The products were characterized by spectroscopic techniques (UV and/or NMR spectroscopy and electrospray or MALDI-TOF mass spectrometry). These studies led to some salient observations with respect to the anticancer activity of Pt(II) complexes **187**−**189**. The monofunctional Pt−S adducts were transformed into Pt−N complexes, in which there was a preferential formation of Pt−N7 bonds. Most of the chelates isolated had Pt−S bonds, and in the case of cisplatin complexes, loss of the ammine trans to sulfur gave rise to the formation of tricoordinate species with platinum mediated peptide−nucleotide cross-links. 1,2-Intrachain platinum GpG adducts were only obtained in very small amounts (1−4%). For example, in the case of cisplatin, only a very small amount of the therapeutic complex **190** was formed on *in vitro* administration of the drug. A large amount of the drug was lost. This observation explained the low therapeutic effect of cisplatin in *in vivo* studies, but this conclusion is based on the assumption that the GpC−cisplatin complex is responsible for the therapeutic effect of cisplatin.

5. Summary and Future Perspectives

In conclusion, we have discussed the research findings related to almost all aspects of the peptide−oligonucleotide conjugates. Section 1 of this review gives a brief introduction to the need for conjugation of peptides to oligonucleotides and their potential applications in diverse fields. We have presented the different POCs known so far in a systematic manner in Chart 1, so as to have a clear understanding on the possible modes of conjugation of peptides to oligonucleotides. Different types of chemical linkages and the means to achieve the same have been summarized in section 2. There are two major synthetic protocols, namely, solid-phase divergent and solid- or liquid-phase convergent methods. For synthesis of oligonucleotides or peptides, the solid-phase divergent method remains indisputably the primary method of preparation, but for preparation of conjugates, the solid-phase sequential method fails miserably due to many factors; important among them are lack of a suitable solid support to assemble both peptide and oligonucleotide sequences one after the other. In this context, we are still far away from an ideal condition, wherein a universal solid support and a uniform protection strategy are used for synthesis of different types of POCs. Also, the new method should be suitable to incorporate all types of amino acids, with no modification, or at least little modification, of the synthetic protocol. This aspect is quite essential, as different amino acids require different side chain protecting groups. Thus, automated high-throughput synthetic protocols for the preparation of conjugates have yet to be realized. There is an urgent need for coordinated efforts by different research groups to attain these goals. Because of these discrepancies, most of the POCs required for biological studies have been prepared by fragment conjugation rather than online synthesis. It is worthy of mention that the biochemical methods for synthesis of conjugates might be given a fair trial in peptide oligonucleotide conjugation chemistry.

The biological properties, for instance, the antisense activity of similar conjugates, are shown to be influenced by the nature of the chemical linkage between the peptide and the oligonucleotide fragments. The disulfide linkage has been chosen in a number of cases owing to its easy reduction in the cellular enzymes. However, its importance has yet to be established beyond doubt. Thus, at present, it may be suggested that the choice of a suitable functional group to link the peptide and oligonucleotide fragments is mostly determined by its synthetic feasibility rather than the biological value.

Initially, the primary reason behind synthesis of these bioconjugates was to enhance the cellular uptake characteristics of the antisense oligonucleotides. The wide number of studies carried out so far continues to prove that the peptide conjugation will soon be a viable alternative to lipid based delivery methodologies. In most cases, the cytosolic entrapment of the conjugates is the crucial step to be surpassed for effective inhibition or modulation of gene expression, especially for those that occur inside the cell nucleus. In the recent past, there have been a few reports on "triggered endosomal release" of the conjugate from such cytosol entrapments, using lysosomotropic reagents such as chloroquine and Ca^{2+} ions.[50,187]

The mechanistic pathways followed by different peptides, for their cellular uptake, still remain to be studied further. However, as pointed out by Gait,[12] without being attached too much to the controversial observations on the mechanism, the research efforts should be focused on finding suitable peptides to internalize the given oligonucleotide to the target cell or subcellular organelle. He has also advocated that the experimentation related to the evaluation of the biological efficacy of the conjugates needs to be simplified and much more data are to be collected. This would help us to understand better the structure−activity relationship of peptides. In addition to antisense oligonucleotides, siRNAs could also be delivered successfully to cells by direct peptide conjugation or using nanoparticles containing peptide sequences. Some of these studies provided insight into the relationship between siRNA cellular uptake, localization, and function, and this would lead to discovery of several new and effective peptide based methods for siRNA delivery. Similarly, peptide conjugation has been proven to be effective in the case of ISS-DNAs as well. Studies on attempts to enhance the cellular uptake characteristics of the plasmid by indirect peptide conjugation have not given any fruitful results so far. The cause of failure needs to be explored further. Nevertheless, the coupling strategy described therein may find applications as a tool for plasmid functionalization.

The concept of the chimeric peptide conjugation approach, namely, attachment of peptides containing two different domains—one for target specificity and the other exclusively for increasing the cellular delivery—may be useful to increase the target specificity of the antisense oligonucleotides without compromising the cellular uptake. The concept of peptide conjugation has also been used to focus on aspects other than mere translocation across the plasma membrane. For example, the influences of peptide conjugation on the binding

affinity to the target nucleic acid, on target specificity, and on intracellular stability have been studied. Similarly, the research papers discussed herein, related to the use of POCs in nanomaterials, and models to study the mechanistic pathways of anticancer agents are in fact the "tip of the iceberg". This reveals that these bioconjugates serve as promising models to explain the anticancer activity of platinum complexes, and this may be extended to other drugs as well. Also, the model studies can be carried out with more complex hybrids and also in the presence of rescue agents to get a deeper insight into their mode of action.

Overall, there are many pitfalls in the way of realizing the full potential uses of these bioconjugates. The future task in this area includes a combination of a straightforward method for chemical synthesis and further advancement in experimental protocols to evaluate the biological effects of conjugates. In particular, developments in the synthesis of the conjugates would in turn expedite research on the applications of these bioconjugates in diverse fields. Nonetheless, the increased number of research reports in the recent past suggests that peptide conjugation to oligonucleotides and their modified analogues is an interesting subject to be pursued further.

6. Glossary

Ac	acetyl
Acm	acetamidomethyl
ACN	acetonitrile
AEEA	2-aminoethoxy-2-ethoxyacetic acid
Ahx	ϵ-aminohexanol
ANT	a 16-amino acid sequence from the *Drosophila antennapedia* protein
ATP	adenosine triphosphate
BAP	bacterial alkaline phosphatase
Bhoc	benzhydryloxycarbonyl
Boc	*tert*-butoxycarbonyl
Bpoc	2-(biphenyl-4-yl)propan-2-yloxycarbonyl
Bzl	benzyl
CAE	capillary array electrophoresis
C.e	*C. elegans*
CEM and CEM-SS	are human T_4-lymphoblastoid cell types originally derived from a child with acute lymphoblastic leukemia and now can be cloned biologically
CMV	cytomegalovirus
CNE	2-cyanoethyl
CPG	controlled pore glass
CpG	cytosine phosphate gunanosine (a dinucleotide)
CPP	cell penetrating peptides
CTL	cytotoxic T lymphocyte
DABCYL	4-[[4-(dimethylamino)phenyl]azo]benzoic acid
dba	dibenzylideneacetone
DCM	dicholoromethane
Dde	1-(4,4-dimethyl-2,6-dioxacyclohexylidene)ethyl
Ddz	2-(3,5-dimethoxyphenyl)propan-2-yloxycarbonyl
DIPEA	*N*,*N*-diisopropylethylamine
Dmab ester	4-{*N*-[1-(4,4-dimethyl-2,6-dioxocyclohexylidene)-3-methylbutyl]amino}benzyl ester
DMAP	4-(dimethylamino)pyridine
Dmbz	2,6-dimethoxybenzoyl
DMF	*N*,*N*-dimethylformamide
DMSO	dimethyl sulfoxide
DMT	4,4′-dimethoxytrityl
dnp	dinitrophenyl
DTT	1,4-dithiothreitol
ED	effective dose
EDC	1-ethyl-3-(3-dimethylaminopropyl)carbodiimide
EGF	epidermal growth factor
ESI	electron spray ionization
FACS	fluorescence activated cell sorting
FBS	fetal bovine serum
Fm	9-fluorenylmethyl
Fmoc	9-fluorenylmethoxycarbonyl
FSCE	free-solution capillary electrophoresis
Ftc	fluoresceinthiocarbamoyl
GFP	green fluorescent protein
GMP	5′-fuanosine monophosphate
IEC	ion-exchange chromatography
IMP	5′-inosine monophosphate
ISS DNA	immunostimulatory sequences of DNA
KDEL	a tetrapeptide (Lys-Asp-Glu-Leu), commonly found at the C-terminus of soluble proteins of the endoplasmic reticulum
LC-MS	liquid chromatography−mass spectrometry
LIF	laser induced fluorescence
LNA	locked nucleic acid
LPFC	liquid-phase fragment conjugation
LTR	long terminal repeat
MALDI	matrix assisted lattice desorption ionization
MAP	multiple antigenic peptide
MBHA	4-methylbenzhydrylamine hydrochloride
MDR-1 (gene)	multidrug resistance (region of gene)
Mmt	4-methoxytrityl
MPP	membrane permeant peptides
MS	mass spectrometry
MTS	membrane translocation signal
Mtt	*N*-methyltrityl- or methylotrityl
NEM	*N*-ethylmaleimide
NHS	*N*-hydroxysuccinimide
NLS	nuclear localization signal
NMP	*N*-methyl pyrrolidinone
NPE	(*o*-nitrophenyl)ethyl ester
ODN	oligodeoxyribonucleotide
PAGE	polyacrylamide gel electrophoresis
PAL	5-[3,5-dimethoxy-4-(aminomethyl)phenoxy]pentanoic acid
PEG	polyethylene glycol
PEGA	poly[acryloylbis(aminopropyl)polyethylene glycol]
PEI	polyethyleneimine
Phac	phenylacetyl
PLL	poly-L-lysine
PMO	phosphorodiamidate morpholino oligomers
PNA	peptide nucleic acid
POC	peptide oligonucleotide conjugates
PPTS	pyridinium *p*-toluenesulfonate
PS	polystyrene
Px	9-phenylxanthyl
PyBOP	(1H-benzotriazol-1-yloxy)tripyrrolodinophosphonium hexafluorophosphate
RGD	Arg-Gly-Asp peptide
RISC	siRNA induced silencing complex
SAX	strong anion exchange (resin)
S-dC28	a 28-mer phosphorothioate oligonucleotide
SEM	standard error of the mean
siRNA	short-interfering RNA
SPDP	*N*-succinimidyl-3-(2-pyridyldithio)propionate
SPFC	solid-phase fragment conjugation
TAR	transactivation region
TAT	trans-activator (domain of HIV-1 gene)
TBDMS	*tert*-butyldimethylsiyl
TEAA	triethylammonium acetate
TFA	trifluoroacetic acid
Tfa	trifluoroacetyl
Tfl	triflyl
TFO	triplex forming oligonucleotide
Th1	a distinct type of T-helper cell responsible for Th1-(immune) response
THF	tetrahydrofuran
TIS	triisopropyl silane

TOF	time-of-flight
Tos	tosyl
TrS	tritylthio
Trt	trityl
VEGF-R2	vascular endothelial growth factor receptor-2

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