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Minireview

Recent advances in oligonucleotide synthesis and their applications

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Short synthetic oligonucleotides are finding wide variety of applications in area of genomics and medicinal chemistry. Since the isolation of nucleic acids to the mapping of human genome, chemical synthesis of nucleic acids has undergone tremendous advancements. Further improvements in this area such as, introduction of high throughput synthesizers, better coupling reagents, improved polymer supports, newer sets of protecting groups for exocyclic amino groups of nucleic bases and introduction of universal polymer supports have completely revolutionized the entire field of nucleic acids chemistry. Most of these developments have been targeted to assemble these molecules more efficiently in a cost-effective manner and rapidly. Preparation of oligonucleotide conjugates has further helped in identifying the newer areas of their applications. A number of conjugates with biological and abiological ligands have been discussed in this article along with their possible wide spectrum of applications. Recently developed microarray technology, which refers to attachment of short oligonucleotides on a solid/polymeric surface, has proved to be useful for screening of genetic mutations, study of polymorphism, as diagnostics, etc. The major developments in these areas are presented in the review.

Keywords: Oligonucleotides, universal polymer support, reusable support, modifications, conjugates, immobilization, microarray.

In the last two decades, the use of synthetic oligonucleotides has increased dramatically. These short oligonucleotides are particularly finding applications in many areas such as DNA sequencing, polymerase chain reaction (PCR), medical diagnosis, and therapeutics (based on antisense, siRNA, anti-protein agents), etc. Such a vast range of applications of these molecules has led to the development of synthetic methods, which can cater to the specific requirements. Last couple of years has seen considerable improvements in the oligonucleotide synthesis chemistry along with polymer supports. The commercial availability of DNA synthesizers and synthons for the synthesis of nucleic acids, including the modified analogs have made the synthesis of good quality oligonucleotides simple and cost-effective. The first ever synthesis of a dinucleotide containing a natural 3'-5' phosphodiester linkage was reported in 1955 by Michelson and Todd[1], who used phosphotriester approach. This triggered the race to develop efficient and rapid methods for the synthesis of oligonucleotides. Subsequently, several approaches were developed and finally, phosphoramidite approach was found to be the most suitable for synthesis of oligonucleotides, which is still in use in conjunction with solid-phase methodology[2]. Then, the introduction of automated gene machines completely revolutionized the entire field of nucleic acids chemistry. Furthermore, with the advent of whole genome sequencing projects, demand of these molecules has risen tremendously. However, to meet the exponentially growing need of synthetic oligonucleotides, the conventional methodology has proved to be somewhat inefficient and costly. Therefore, efforts are afoot to develop economical and rapid protocols to produce these molecules in

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Abbreviations: APS, ammonium persulfate; *t*-Boc, *tert*-butyloxycarbonyl; DMAP, 4-dimethylaminopyridine; DBU, 1,8-diazabicylo [5.4.0] undec-1-ene; FAS, ferrous ammonium sulfate; HBTU, *O*-benzotriazol-1-yl-N,N,N',N'-tetramethyluronium hexa-fluorophosphate; HOBT, 1-hydroxybenzotriazole; LCAA-CPG, long chain alkylamine-controlled pore glass; ODN, oligodeoxyribonucleotide; PCR, polymerase chain reaction; PEG, polyethylene glycol; PLGA, poly (D, L-lactic-co-glycolic acid); PNA, peptide nucleic acids; POCs; peptide-oligonucleotide conjugates; siRNA, small interfering ribonucleic acid; TFA, tri-fluoroacetic acid; TEMED, N,N,N',N'-tetramethylethy-lenediamine.

relatively shorter times, without compromising the quality. In this review, we present the efforts made in this direction in recent years, focussing mainly on the following: (i) cost-effective methods for oligonucleotide synthesis; (ii) preparation of oligonucleotide conjugates; and (iii) microarray (biochip) technology — a prominent tool for diagnostic purposes.

(i) Cost-effective Methods for Oligonucleotide Synthesis

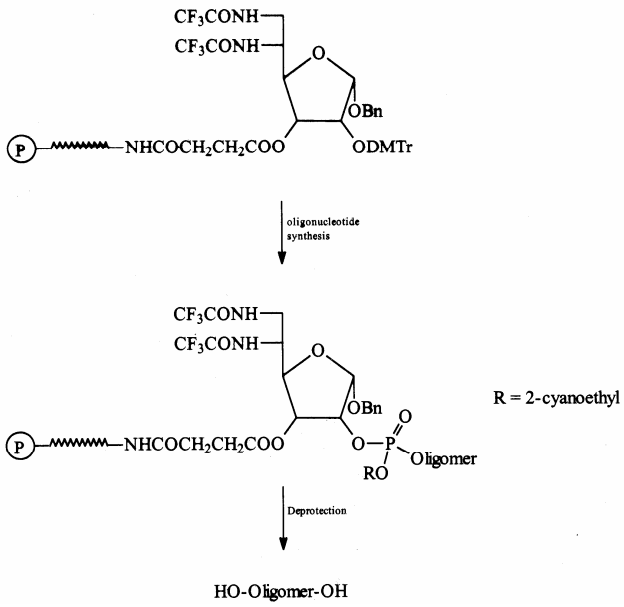
Attempts made to develop cost-effective methodologies for synthesis of a large number of oligonucleotides and at large-scale have been categorized in the following headings: (a), development of universal polymer support; (b), reusable/recyclable polymer supports; (c), synthesis of multiple oligonucleotides on a single polymer support; and (d), large-scale synthesis of oligonucleotides.

(a) Universal polymer supports for oligonucleotide synthesis

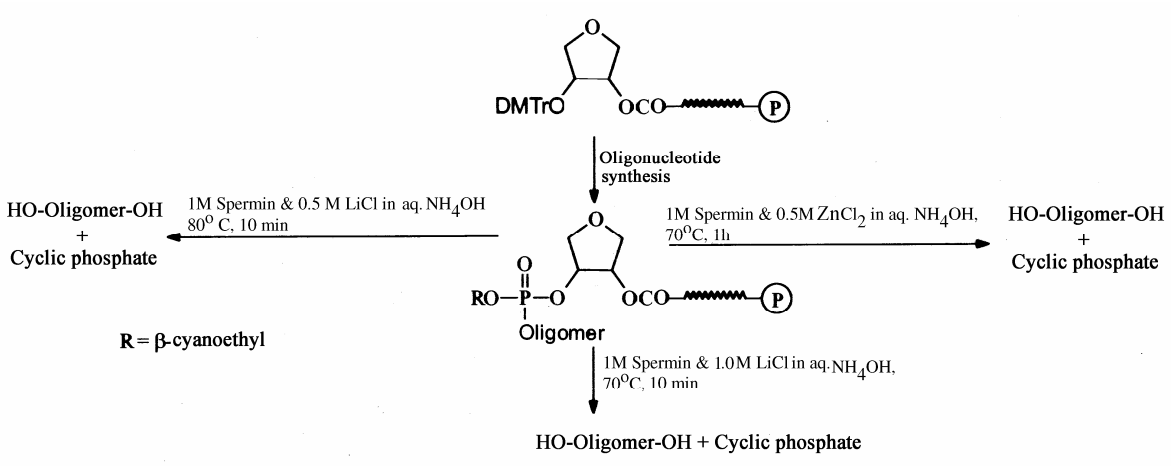
Conventionally, solid-phase synthesis of oligonucleotides requires the use of a leader nucleoside attached polymer support[2]. Therefore, to synthesize these molecules, at least eight polymer supports are required, four for oligodeoxyribo- (dA, dC, dG and T) and four for ribonucleotides (rA, rC, rG and U). The number of supports may increase, if one wants to employ different types of synthons (conventional/labile/modified) for the synthesis of oligonucleotides and their modified analogs. At present, more than 50 polymer supports are available from commercial sources. In order to cut-short the time required to prepare such a large number of pre-derivatized polymer supports, the need was felt to develop a universal polymer support that could be used for carrying out all types of oligonucleotide syntheses. This strategy offers some distinct advantages over the conventional one: (i), preparation of nucleosidic supports can be avoided; (ii), contamination of supports can be eliminated; (iii), error in selection of supports can be minimized in 48 or 96 well synthesizers; and (iv), 3′-modified nucleosides can be employed as phosphoramidites. The first ever effort in this direction was made by Gough *et al.*[3], who synthesized a universal polymer support based on uridine nucleoside. Since then a number of universal polymer supports based on nucleosidic and non-nucleosidic linkers as well as rapid deprotection conditions for the cleavage of oligomer chains from them have been proposed[2,4,5].

In an alternative approach, recently a new universal support, incorporating an ethylenediamine moiety in ribose-like linker to enhance the cleavage of terminal phosphodiester along with the sugar, linked to polymer support (**Scheme 1**) has been proposed[6]; the idea of incorporating an ethylenediamine residue was derived from an earlier report[7] in which the role of di- and oligoamines on the hydrolysis of RNA under physiological conditions was studied. In addition, the cleavage of hairpin and hammerhead ribozymes by a polyamine in the presence of Mg^{2+} is also reported[8]. As the cleavage of oligomers from a *cis*-diol bearing universal polymer support resembles base-catalyzed hydrolysis of RNA, Kumar *et al.*[9] carried out a detailed study of the role of polyamines in conjunction with metal ions, for achieving a rapid generation of fully deprotected oligonucleotides from universal polymer supports (**Scheme 2**). They discussed the effect of various metal ions (such as nickel, cobalt, copper, zinc and lithium) as well as temperature in conjunction with amines or oligoamines and demonstrated that fully deprotected oligonucleotides can be obtained in just 10 min at 80°C, using 1 *M* spermine in presence of 0.5 *M* lithium chloride.

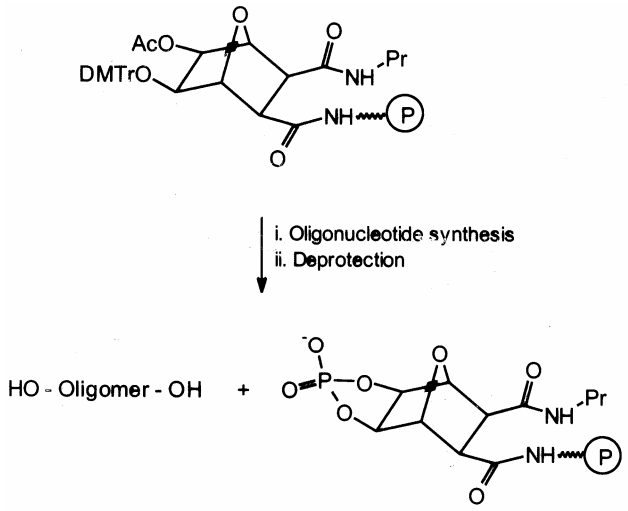
Very recently, a novel conformationally pre-organized non-nucleosidic universal solid support for



Scheme 1 — Azhayev's universal polymer support (ref. 6)



Scheme 2 — Polyamine-assisted cleavage of oligonucleotide chains from *cis*-diol based universal support (ref. 9)

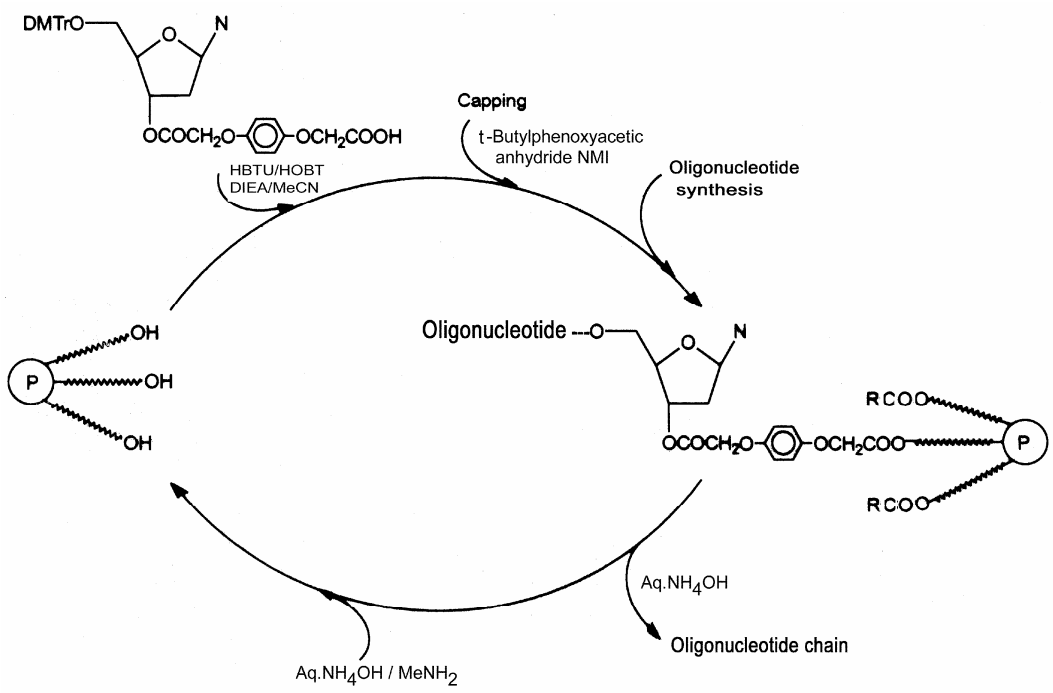


Scheme 3 — Conformationally pre-organised non-nucleosidic universal support (ref. 10)

oligonucleotide synthesis **(Scheme 3)** has been reported[10]. The solid support consisting of two chemically equivalent hydroxyl groups locked in *syn*-periplanar orientation and orthogonally protected with 4,4'-dimethoxytrityl and acetyl groups was extensively tested for the preparation of oligonucleotides and their phosphorothioate analogues. Upon completion of oligonucleotide chain assembly, conc. NH_4OH treatment at room temperature yielded oligonucleotide material in solution.

(b) Reusable/recyclable polymer supports

Solid-phase oligonucleotide synthesis comprises three components, viz., polymer support, appropriately protected nucleoside-phosphoramidite synthons and solvents and reagents, which account for 40, 30 and 30%, respectively of the total cost to produce them. Therefore, the cost of production of these molecules could be checked, if one could design a strategy by which the cost of the polymer supports be taken care of. An effective way to address this problem is to employ polymer support that can be reused for a few synthesis cycles. In this regard, Pon *et al.*[11] demonstrated the reusability of controlled pore glass (CPG)-based support, the most commonly employed support for routine synthesis. They generated hydroxyl groups on the polymer support and Q-linker (hydroquinone-*O*, *O'*-diacetic acid) attached through an ester linkage. On the other end, an appropriately protected nucleoside has been coupled, using *O*-benzotriazol-1-yl-N,N,N',N'-tetramethyluronium hexafluorophosphate (HBTU) and 1-hydroxybenzotriazole (HOBT) as activating reagents and then this support was used for synthesis of oligonucleotide chains **(Scheme 4)**. They employed labile capping reagents to get fully deprotected hydroxyl groups on the support after achieving deprotection of oligonucleotides. Therefore, the cleavage of the oligomer from the support with aq. NH_4OH generated partially deprotected hydroxyl support, which on treatment with NH_4OH-methylamine gave rise to fully deprotected hydroxyl support for another cycle. In this way, they demonstrated synthesis of six different oligonucleotides and 25 cycles of nucleoside derivatization and cleavage performed on the reusable support. The methodology would further be of great impact for repeated synthesis of therapeutic



Scheme 4 — Oligonucleotide synthesis on a recyclable polymer support (ref. 11)

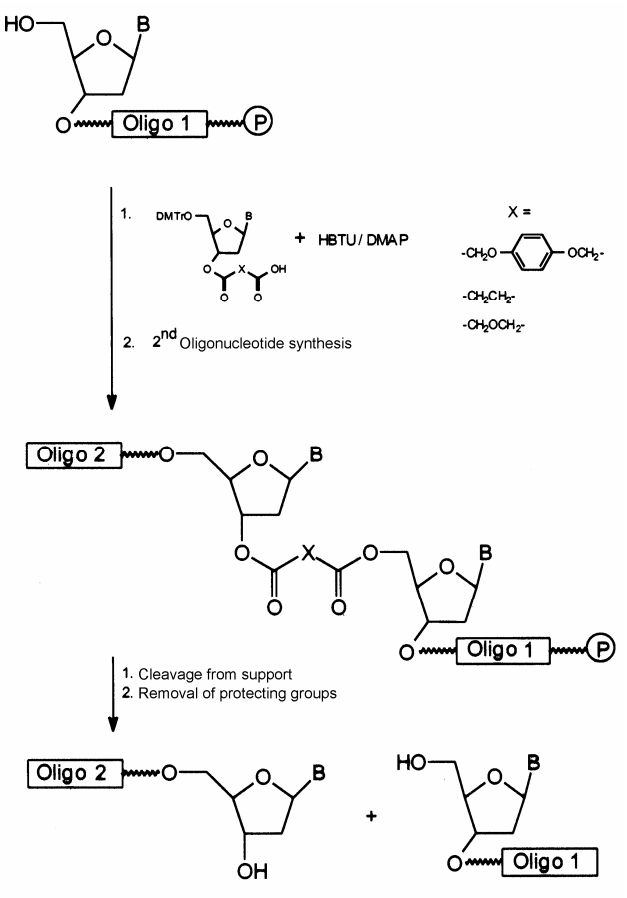
oligonucleotides on large-scale, if organic polymer supports could be employed, because silica-based supports have a tendency towards deformation under alkaline conditions.

(c) Tandem synthesis of oligonucleotides on a single polymer support

In this methodology, the polymer support can be used more effectively to assemble oligonucleotides in a cost-effective manner. This can meet the requirement of oligonucleotides both on small and large-scales. The method provides an opportunity to assemble sets of oligonucleotides for PCR, multiplexed genotyping primers and double stranded oligonucleotides, which are used together. Similarly, for large-scale synthesis, multiple copies of an oligomer can be assembled, which would ultimately reduce the cost of the oligomer. Originally, this concept was evolved by Hardy *et al.*[12] to synthesize two different oligomers (two oligomers per synthesis, TOPS) or multiple copies of an oligomer in one continuous solid-phase synthesis. However, time required to cleave the oligomers was a bit longer and conditions were too harsh and sometimes resulted in non-quantitative dephosphorylation, which actually overweighed the utility of the concept. Recently, in a modified version of this concept, Pon *et al.*[13,14] employed linking reagents (either as their 3′-carboxylates or as linker-phosphoramidite reagents) with rapidly cleavable 3'-ester linkage. Carboxylates are coupled by using HBTU/DMAP condensing reagent and linker-phosphoramidite is attached in a manner analogous to the coupling of the normal nucleoside-phosphoramidites (**Scheme 5**). Similarly, they demonstrated that oligonucleotides in large scale could also be assembled synthesizing them repeatedly on the same support separated by a cleavable linker between two oligonucleotide sequences.

(d) Large-scale synthesis of oligonucleotides

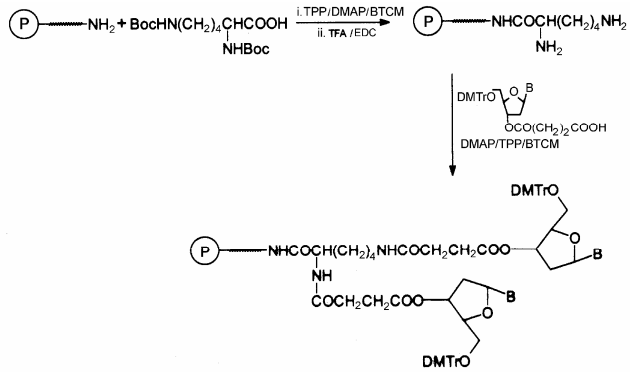
A part of this section has already been discussed in above sections. Here, the alternative approaches have been taken up for synthesis of oligonucleotides at large-scales. Scaling up of oligonucleotide synthesis is an important area, as it has been recently predicted that antisense oligonucleotides of pharmaceutical importance would be required in quantities of several tonnes, annually. To meet this huge demand, some research groups have proposed strategies for large-scale synthesis of oligonucleotides. Mainly, this problem can be addressed by two ways: (i) to employ commercially available high loading polymer supports, such as highly cross-linked polystyrene,



Scheme 5 — Tandem synthesis of multiple oligonucleotides (refs. 13,14)

tenta-gel, Merckogel and soluble supports, such as polyethylene glycol for the synthesis purposes; and (ii) to design protocols, by which loading of functional groups on the routinely used existing polymer supports can be increased.

In one of such reports, Patnaik *et al.*[15] reported the preparation of high loading long chain alkylamine-controlled pore glass (LCAA-CPG) support (**Scheme 6**). They coupled di-Boc-protected lysine to LCAA-CPG and subsequently, after removal of protecting groups, appropriately protected nucleoside-3'-*O*-succinates were coupled. They showed about two-fold increase in the loading of the functional groups (NH_2 groups as well as nucleoside loadings); however, attempts to further increase its loading were unsuccessful. In yet another approach, Manchanda *et al.*[16] used a well-known polymerization reaction (involving acrylamide) to increase the amino group loading on the CPG supports with variable pore sizes. The method involved the generation of acryloyl



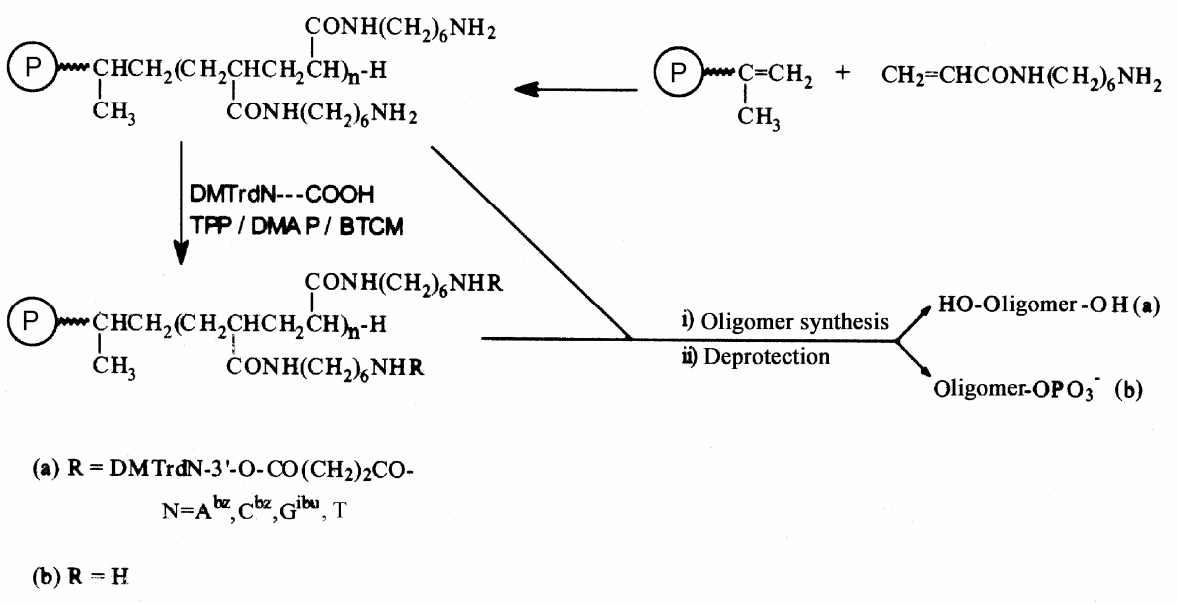
Scheme 6 — Synthesis of oligonucleotides on a high loading polymer support (ref. 15)

groups on the polymer supports and the preparation of N-6-aminohexylacrylamide (**Scheme 7**). Mixing of these two in presence of TEMED/FAS/APS generated high loading of amino groups on polymer supports. In both approaches, the quality of synthesized oligonucleotides was comparable to oligonucleotides synthesized on standard supports. The methods seem to be quite useful for improving loading on the other commercially available high loading supports, such as Tenta gel, Merckogel, etc.

(ii) Preparation of Oligonucleotide Conjugates

During past two decades, considerable attention has been paid for the development of oligonucleotide conjugates to determine their real therapeutic value. The need to prepare these conjugates was felt only after finding the limitations of normal oligonucleotide to act as an ideal therapeutic molecule. Thus, these conjugates came into existence to improve certain properties such as cell-specific delivery of these molecules, cellular uptake, intracellular distribution, resistance against nucleases, binding strength, target specificity and mechanism of target inactivation, have to be made. The conjugates could be prepared with specific ligands/molecules of biological or abiological importance[17-24]. Currently, several such synthetic oligonucleotide conjugates are being studied for their potential diagnostic and therapeutic applications[25,26].

To prepare oligonucleotide conjugates, the most commonly used positions to incorporate the ligands are 5'- and 3′-termini of oligonucleotides[27,28] as modifications at these sites are the simplest and do not interfere in Watson-Crick hydrogen bonding and hence do not affect the stability of the duplex formed between two complementary oligonucleotide sequences. The modifications at 5'-terminal are more

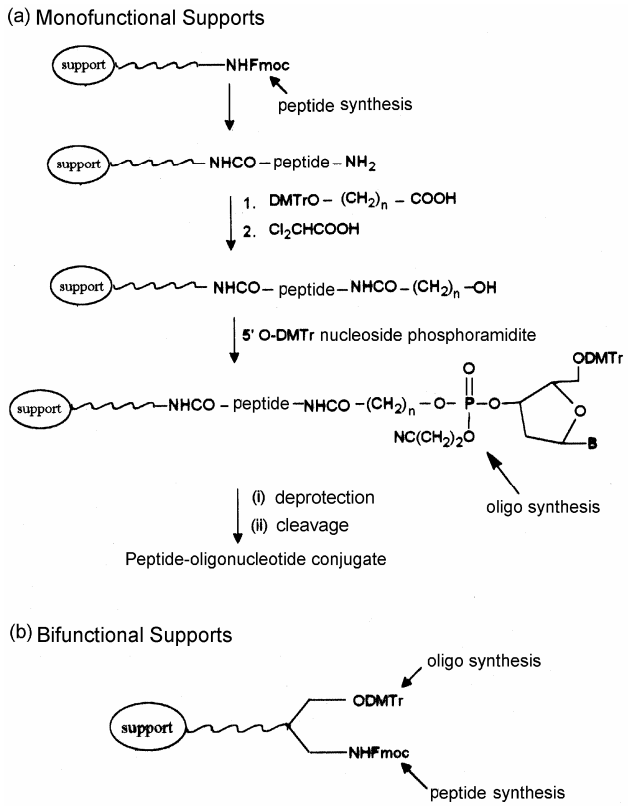


Scheme 7 — High loading supports for the synthesis of oligonucleotides (ref. 16)

advantageous as they can be introduced in the machine itself during the synthesis cycle. On the other hand, 3′-terminal modifications are somewhat difficult to achieve, as these days, most of the syntheses are being carried out on solid supports and 3′-hydroxyl function is inaccessible for the desired modification to be incorporated. Moreover, 3′-hydroxyl group is not sufficiently nucleophilic for introducing modifications during post-synthesis work-up. Therefore, engineered supports are required with a specific modification at this terminal, which during final deprotection step yield oligonucleotides with desired nucleophilic or electrophilic group or a specific ligand attached to them. Some ligands that have been attached to supports, in such a manner, are cholesterol, carboxyfluorescein, biotin, and iodophenylacetyl etc.[29] Preliminary accounts of these modifications have already been covered in the previous reviews[2,30]. In the current account, conjugates of biological importance have been taken up with the possibility of being potential therapeutic agents.

(a) Peptide-oligonucleotide conjugates

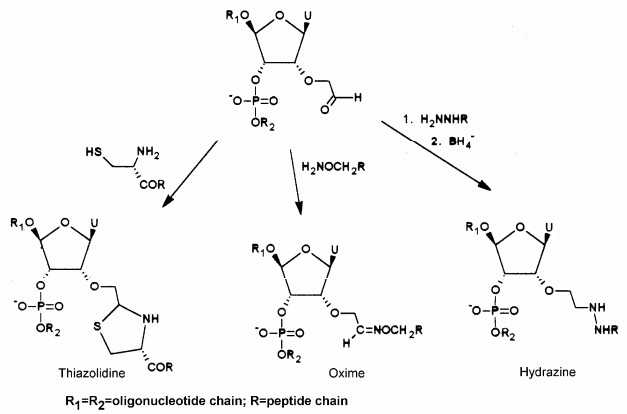
Peptide-oligonucleotide conjugates (POCs) constitute one of the most important classes of oligonucleotide conjugates. They are of great value not only to enhance their resistance to nucleases and cellular uptake, but also for some specific functions imparted by peptide part of their molecule. Basically, these conjugates are prepared by solution phase method or solid-phase method; however, due to some advantages, needless to mention here, they have been prepared, using solid-phase synthesis[31,32] on CPG support, macroporous polystyrene beads, and teflon etc. Solid-phase assembly of these conjugates is carried out by two methods: in-line synthesis and fragment conjugation. In the former, the peptide and oligonucleotide are synthesized sequentially on an automatic synthesizer as shown in **Scheme 8**. The first report on solid-phase synthesis using in-line method was published by Haralambidis *et al.*[33] using standard 9-fluorenyl methoxy carbonyl (Fmoc) and phosphoramidite chemistries on a CPG support to assemble peptide and oligonucleotide parts, respectively. A ω-hydroxycarboxylic acid was used to convert amino group at N-terminus of the peptide into -OH group, which then served as a linker between the peptide and oligonucleotide components. In the end, TFA/ethanedithiol was applied to deprotect the peptide and then aqueous ammonia treatment was used to simultaneously deprotect the oligonucleotide and cleave the conjugate from the support. To avoid harsh treatment with TFA, some base labile protecting groups for the side chains of peptides were introduced[34]. Subsequently, other modifications[35-37], including linkers having two different functional groups were incorporated to further simplify the preparation of POCs.



Scheme 8 — In-line solid-phase assembly of peptide-oligonucleotide conjugates (ref. 33)

In a slightly different approach, Bergmann *et al.*[38] prepared a conjugate containing oligonucleotides (DNA-peptide-DNA) on either sides of a peptide. The first oligonucleotide was synthesized on sarcosine modified CPG, using phosphoramidite chemistry and at the end of the oligonucleotide, 5'-amino-thymidine was incorporated to have an amino group for subsequent peptide synthesis. Again phosphoramidite chemistry was used for the second synthesis. Cleavage from support and removal of protecting groups were achieved using aq. NH_4OH.

In fragment conjugation, the peptide and oligonucleotide fragments were synthesized separately, cleaved from their respective solid supports, deprotected, purified separately and then coupled, using a suitable condensing/bifunctional reagent **(Scheme 9)**. The approach came into existence, when it was realized that the reagents used in peptide chemistry might be too strong for the oligonucleotides and *vice-versa*. The method requires generation of reactive groups on the moieties, oligonucleotides and peptides. Some of such linkages are shown in Table 1. Using this approach, Zatsepin



Scheme 9 — Post-synthesis conjugation strategy for the preparation of POCs (ref. 45)

Table 1 — Linkages used in fragment conjugation in the preparatio of POCs

Functional group on		Linkage	Ref.
Peptide	Oligonucleotide		
N-α-bromoacetyl	Thiol	Thioether	39
Maleimido propanoate	Thiol	Thioether	40
Maleimide	Cysteine	Thioether	41
Thioester	Cysteine	Thioether	41,42
Oxyamine	Aldehyde	Oxime	43
Acylated with cysteine-peptide	Aldehyde	Thiazolidine	43
Aldehyde	Oxyamine	Oxime	43
ε-Amino (lysine)	Succinylate	Amide	44

et al.[45] synthesized POCs with single or multiple peptides in good yield, using hydrazine linkages. The method had several advantages as it was possible to attach more than one peptide at defined nucleoside residue locations, the binding of the oligonucleotide to RNA structures was not affected significantly and the facile mild conjugation reaction types do not require large excess of peptide reagents. In a slightly different approach, Bruick *et al.*[46] reported template directed ligation, in which pre-purified, unprotected peptide with a carboxy terminal thioester was temporarily tethered to a 5′-thiol terminated oligonucleotide through a thioester linkage. This thioester linked intermediate and a second oligonucleotide which had a 3′ amino group were simultaneously hybridized to adjacent sequences on a complementary template, whereupon the peptide migrated from the first to the second oligonucleotide, thereby forming a stable amide linkage with the latter.

Most of conjugation reactions have been carried out in a mixture of aqueous and organic solutions, due

Table 2—Peptides used in the preparation of POCs

Peptide	Function	Ref.
CGGSPKKSPKK	DNA binding	64
CAAKKAAKKAAKK	DNA binding	64
CTPKRPRGRPKK	DNA binding	65
KKAAKKACAKKAAKK	DNA binding	65
AAKRVKLG	Delivery	46
TQPREEQYNSTFRV	Delivery	66
YGEEDTSEKDEL	Delivery	39
GGH	Artificial nuclease	27
(LKKL) 3	Artificial nuclease	34

to diverse nature of oligonucleotides and peptides. In spite of this, when the peptides, that are highly cationic, interacted with oligonucleotides, which are highly anionic, there occurred precipitations. This problem, however, has been overcome by employing a high concentration of salt in the reaction mixture[47,48].

POCs have shown promising applications, particularly, in the area of antisense therapy[49], where interference with the expression of target genes is achieved in a highly selective manner, by using a complementary or antisense sequence. Antisense oligonucleotides[50,51] are short sequences of synthetic single stranded DNA usually less than 30 nucleotides in length, and complementary to a specific intracellular target, normally mRNA. They could also form triple helices with double stranded DNA or interact with proteins affecting gene expression at different steps. This selective inhibition of expression of specific genes by antisense oligonucleotides provides an attractive and elegant approach to drug discovery[50-54]. Some of them, in particular CpG-oligonucleotides have been found to act as immunostimulators[55,56]. They also have a tendency to enhance the antitumour efficacy of some peptide vaccines[57]. Therefore, antisense strategies can be used for any gene, for which sequence information is available. Recently, small interfering RNAs (siRNA), which are double stranded have been found to offer efficient antigene strategy for gene suppression[58, 59].

In antisense therapy, only 1 to 2% of the oligonucleotides were found to reach the cell in about 4 hr. In order to overcome the problems related to their poor uptake and nucleolytic degradation, modified oligonucleotides with increased lipid solubility and stability have been introduced[60]. These modifications were incorporated in backbone, base and sugar of synthetic oligonucleotides[61,62]. To address the problem of cellular delivery and to improve its efficiency, POCs as well as peptide-lipid vector[63] (composed of lipofectin, an integrin-targeting peptide and DNA) have been prepared. Some of the most commonly used peptides in preparation of POCs for this purpose are given in Table 2.

The importance of peptide in improving the role of nucleic acids can be felt from the development of peptide nucleic acids (PNA)[67,68] which are analogs of DNA, in which the backbone is a pseudopeptide instead of a sugar. With these, new applications in the field of molecular biology have emerged, particularly where the purpose could not be served by simple oligonucleotides.

(b) Conjugates with Intercalating Agents

A short antisense or antigene oligonucleotide might not have a strong enough affinity towards its target sequence. This affinity could be increased markedly by covalently linking an intercalator at the end of the oligonucleotide chain[69]. Acridine and its derivative, 9-amino-6-chloro-2-methoxy acridine are reported to form heteroduplex with RNA, thus activating specific phosphodiester linkages in the RNA; as a result, these linkages are selectively hydrolyzed by lanthanide ion[70]. Gottikh *et al.*[71] developed methods of intercalator incorporation at 5' and/or 3' terminal positions or one of the pyrimidine heterocyclic bases, with a view to design short "switch" oligonucleotides capable of inhibiting selectively HIV integration. The inhibition induced by intercalators is a passive one as they can be dissociated from the complementary sequence. For causing irreversible damage to the target sequence, cross-linking and cleaving reagents, such as alkylating agents[72] and psoralen[73,74] have been conjugated to oligonucleotides, so that permanent block of the gene expression can be achieved.

(c) Miscellaneous Conjugates

Triple helix forming oligonucleotides

Direct interference with the gene expression or viral replication can be achieved by targeting DNA with oligonucleotides that bind in a sequence specific manner in the major groove of the duplex target to form a triple-helix; this is possible by way of Hoogsteen base pairing. Triple-helix formation is advantageous over the classical antisense approach (where mRNA is targeted), as in this case only lesser number of molecules need to be inactivated to inhibit expression as many copies of mRNA are produced from one equivalent of DNA. Oligonucleotide conjugates with carminomycinone derivatives[75] have

also been prepared to study their triple-helix forming tendency with the polypurine tract present in the human integrated genome HIV-1 infected cells. Steroid-DNA conjugates capable of forming triple-helix have also been prepared[76]. Amsacrine-4-carboxamide oligonucleotide conjugates were found to modulate the extent of DNA cleavage of topoisomerase II.[77]

Ribozyme mimics

Ribozymes are enzymes responsible for cleavage and ligation of specific phosphodiester bonds within RNA molecules. An extension of the antisense approach is the development of systems that can cleave the target mRNA substrate. Mimics of ribozymes have been prepared to this effect. These are conjugates of oligonucleotides with compounds such as Cu (II)-serinol-terpyridine[78] and derivatives of phenanthroline[79]. Di-imidazole-derived constructs of peptides have also been used for synthesizing ribonuclease mimics[80].

Polymer conjugates

In order to improve the sensitivity of an enzyme-linked oligosorbent assay diagnostic test, Minard-Basquin *et al.*[81] prepared conjugates of oligonucleotides with poly (maleic anhydride-alt-ethylene). Conjugates with polymer have also been used for the delivery of oligodeoxyribonucleotides into cells. Micelles from conjugates of biodegradable poly (D, L-lactic-co-glycolic acid) (PLGA) with oligodeoxyribonucleotides (ODNs) have also been prepared[82]. These conjugates self-assemble in aqueous solution to form a micellar structure, where PLGA segments serve as a hydrophobic core and ODN segments as a surrounding hydrophilic corona. These micelles can release ODN in a sustained manner by controlled degradation of hydrophobic PLGA chains.

Photoreactive conjugates

Conjugates with anthraquinone have been prepared with a view to develop electrical sensors for DNA molecules[83,84]. Pyrene conjugated oligonucleotides are also expected to play a similar role[85]. Some such conjugates find use in the preparation of oligonucleotide arrays[83].

Photoresponsive conjugates

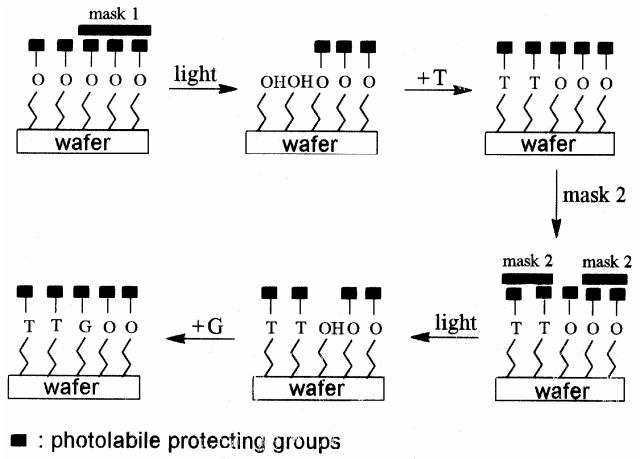
In the past few years, interest has also developed in the area of photoresponsive oligonucleotides that alter the duplex forming activity in response to photostimuli and can be involved in photoregulation of bioreactions. Moieties such as stilbene[86] and azobenzene[87] have been incorporated into the side chain of oligonucleotides. On irradiating with light, the stability of double stranded structure of the modified oligonucleotide is altered due to the interconversion of geometrical isomers of the ligand[88-90]. Photoregulation of DNA triplex formation in the presence of "azo" moiety in the oligonucleotide, have also been reported[91,92]. Even conformationally locked nucleosides have been prepared[93].

(iii) Oligonucleotide Arrays (Biochip)

DNA microarray is a powerful tool[94-100] that allows simultaneous detection of many different target molecules present in a sample. It consists of systematically arranged DNAs on a suitable solid support. The major applications of this technique are the large scale screening of mutations[101,102] and studies of gene polymorphism[94], gene expression analysis[94-96], disease diagnosis[97], identification and characterization of pathogens[99] etc. Currently, most of the microarrays are being prepared by using PCR DNA products. The efficiency of the array depends mainly upon sequence of the capture probes and the way they are fixed onto the surface. In other words, the technique involves the covalent fixing of known oligonucleotide sequences at the discrete locations on the surface of choice. In expression profiling experiments, using cDNA microarrays, several discrete DNA sequences are spotted onto a glass microarray slide and subsequently hybridized to fluorescently labeled cDNAs. The most commonly used fluorescent dyes are Cy3, Cy5,[103,104] Hex, Fam, Joe, Tamra, Rox and Tet[105-108]. The term 'oligonucleotide array' refers to the systematic arrangement of oligonucleotides on a solid or polymer support. For making oligonucleotide arrays, several surface materials viz., nylon, nitrocellulose, glass, polyacrylamide, polypropylene, polystyrene, silicon, teflon, optical fibres, gold, polypyrrole, and poly(ethylene glycol) [PEG] grafted on silica surfaces etc. have been tested[109,110]. But, glass and polypropylene are the most commonly used materials as they can easily be modified to generate functional groups, however, glass is considered to be the best as it can be used in laser scanners as it offers low background.

Currently, two distinct approaches are being followed for the construction of oligonucleotide arrays. The first one is based on photolithographic

technique[111] which involves direct *in situ* synthesis of oligonucleotides at the pre-determined sites on the solid or polymeric surface, using photolabile protecting groups instead of conventional one at 5′-end of the nucleosides as shown in **Scheme 10**. The hydroxyl groups are generated on the surface and blocked by photolabile-protecting groups and then the surface is exposed to ~UV light through a photolithographic mask to generate a pattern of free hydroxyl groups on the surface. These hydroxyl groups react with photoprotected nucleoside-phosphoramidites, according to phosphoramidite chemistry. A second photolithographic mask is then applied and the surface exposed to UV light to generate second pattern of hydroxyl groups, followed by coupling with 5′-photoprotected nucleoside-phosphoramidite. Likewise, patterns are generated and oligomer chains are extended. Using this technique, a density of 10^6 sequences/cm^2 has been achieved on a biochip. To make this method more attractive, several research groups have introduced photolabile-protecting groups[112-115], which can be removed cleanly and rapidly from the 5′-hydroxyl functionalities. Basically, the lability of a photocleavable group depends on the wavelength and polarity of a solvent employed. Success of this method relies upon three factors, viz., accuracy in alignment of the masks, efficiency of removal of photo-protecting groups and the yields of the phosphoramidite coupling step. The major advantage of this synthesis is that the density of synthesized oligomer per spot can be monitored by adjusting loading of the leader nucleoside on the surface of



Scheme 10—Construction of oligonucleotide arrays using photolithographic technique (ref. 111)

synthesis. Parallel synthesis of oligonucleotides by this method is found to be the most successful approach for making high-density oligonucleotide arrays[116]. Using this technique, arrays have been synthesized on glass, optical fibers and polypropylene films. However, major drawbacks of this method are the requirement of expensive photomasks, which control the light emission on the surface, sophisticated instrumentation and contamination of desired length oligonucleotides with truncated molecules.

Very recently, in an alternative approach, Albert *et al.*[117] demonstrated light directed synthesis of high-density microarrays in 5′-3′ direction. Thus, parallel genotyping and sequencing can be done on the array surface, because 3′-end is available for enzymatic reactions, such as sequence specific primer extension and ligation reactions. For achieving complete deprotection of photoprotected 5′-OH groups, Beier and Hoheisel[118] recommended the use of base-assisted photo-deprotection of NPPOC (2-(2-nitrophenyl) propoxy carbonyl). They employed 0.05 *M* DBU in acetonitrile as a deprotecting reagent under UV light and reported a marked 12% increase in coupling efficiency per condensation.

In another approach, to overcome the limitations, to some extent, of photolithographic technique, oligonucleotides arrays on an epoxy coated glass surface, using non-labile linkers with terminal hydroxyl groups have been prepared[119]; however, the problem related to contamination of oligonucleotides with shorter truncated oligonucleotide sequences could not be addressed. The use of exonucleases has been suggested for this purpose[120] as they remove any surface bound oligonucleotide lacking a 5′-terminal blocking function, *in situ.* However, the strategy does not work in fragments shorter than 10 bases. For this purpose, an alternative strategy in which reactive functions at 5′-end of the oligonucleotide were permitted to react with functions on support before 3'-end is released was adopted[121].

The second method of preparing oligonucleotide arrays relies on the post-synthesis immobilization of oligonucleotides. Conventionally, gene analysis was performed by hybridization of labeled probes to DNA targets that were non-covalently adsorbed to solid supports. Such DNA films were susceptible to removal from the surface under the conditions used for hybridization. Therefore, covalent bindings are preferred and DNA is cross-linked by UV irradiation

to form covalent bonds between thymidine residues in the DNA and positively charged amino groups added on the functionalized slides[122]. However, location and the number of fixation sites of DNA and hence the length of sequences available for hybridization varies with fixation conditions. An improvement has been made by fixing DNA through the extremities. Most of the immobilization reactions involve the attachment of electrophilic/nucleophilic glass surfaces with nucleophilic/electrophilic oligonucleotides. In this method, individual oligonucleotides may be synthesized separately, purified and then they can be immobilized at defined sites on a solid surface. This method is preferred over the first one, because it provides the flexibility in the sense that a variety of biomolecules, viz., oligonucleotides, peptides, PNA and proteins, etc., can be covalently fixed up on the surface of choice. A number of methods have been reported for the post-synthesis immobilization of oligonucleotides on a variety of surfaces (Table 3).

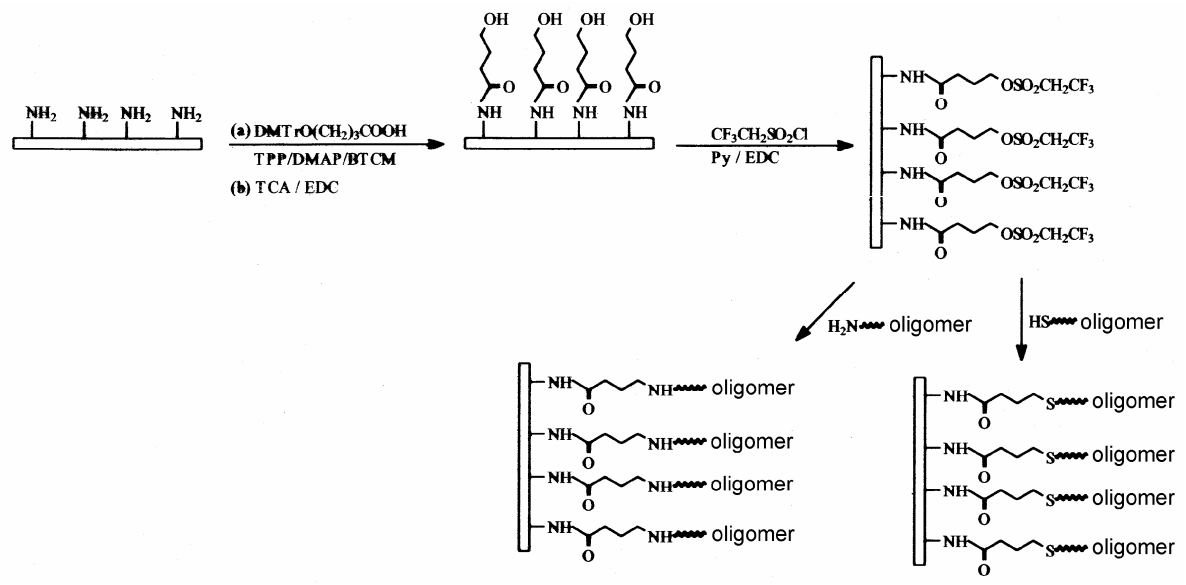
In an elegant approach, hydrazide modified oligonucleotides[141] which can react with a number of functionalized surfaces, such as aldehydic surfaces in the presence of a reducing agent, dialdehydic surfaces, acyl halidic surfaces in the presence of suitable acid scavengers, carboxylic acid surfaces in the presence of suitable condensing reagents, activated carboxylic acids surfaces, viz., *p*-nitrophenyl or N-hydroxysuccinimde esters etc. have been developed. Moreover, with lower p_{Ka} value (4-5) of hydrazides, as compared to primary amine (10-11), they remain reactive at neutral to slightly acidic conditions.

In a reversal of above method, surface can also be modified with the hydrazide group[142]. Here, benzaldehyde modified oligonucleotides can be attached to semicarbazide modified glass to generate semicarbazone-linked oligonucleotide arrays.

Very recently, Kumar and Gupta[143] developed a simple method to construct oligonucleotide array on a variety of surfaces, using commonly available reagents and chemistry with good efficiency and accuracy. The method involves the generation of hydroxyl functionalities, followed by their activation with tresyl chloride (**Scheme 11**). The activated surface in the subsequent reaction is used to covalently immobilize oligonucleotides having mercaptohexyl- or aminohexyl functionalities to create oligonucleotide array. The constructed oligonucleotide arrays were successfully used to analyze oligonucleotides by hybridization technique. In yet another approach, Strother *et al.*[137] described a method for attaching the oligonucleotides to silicon surfaces, which were functionalized with *t*-Boc protected 10-aminodec-1-ene under the influence of UV light. After attachment, *t*-Boc group was removed and the resulting amino groups were coupled to thiolated oligonucleotides, using a heterobifunctional

Table 3 — Immobilization of oligonucleotides

Support	Functional group on support	Modification on oligonucleotide	Ref.
Glass	Thiol	5'-Disulfide	123
Glass	Isothiocyanate	Amine	124
Glass	Aldehyde	Amine	125
Glass	Mercaptoalkyl	Maleimide	126
Glass	Bromoacetamide	Phosphorothioate	127
Glass	*p*-Aminophenyl	Carboxyl	128
Glass/Silicon	Epoxide	Amino	129,130
Glass or polypropylene	N-hydroxysuccinimidyl-esters or imidoesters	5' or 3'-Amino	112
Silanized glass	Amino	5'-Thiol	131
Polyacrylamide on glass	Amino or aldehyde	3'-Amino or aldehyde	132
Glass coated with polyethylenimine	Cyanuric chloride	Alkylamino	133
Plastic	Acrylic groups	5'-Acrylamide	134
CPG, polystyrene-divinylbenzene, sephacryl, chondroitin	Carboxylic, alkylamino on CPG	5'-Aminoalkyl or 5'-phosphorylated	135
Silicon	Maleimide	Thiol	136,137
Quartz/Gold	Maleimide	Thiol	138, 139
Gold	Disulfide	Thiol	140



Scheme 11 — Construction of oligonucleotide arrays using post-synthesis immobilization strategy (ref. 143)

crosslinker, SSMCC (sulfo-succinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate) to generate oligonucleotide arrays. The density of immobilized oligonucleotides was controlled by varying the attachment of *t*-Boc-10-aminodec-1-ene and partially allowing the deprotection of *t*-Boc group from *t*-Boc-10-aminodec-1-ene.

It has been observed in studies related to construction of oligonucleotide arrays that glass surfaces coated with strongly electrophilic surfaces possess poor shelf life; hence, surfaces with groups such as aminoalkyl and mercaptoalkyl have mostly been used for this purpose[125]. In a recent method, the attachment of an oligonucleotide modified with phosphorothioates in its backbone to a glass surface with bromoacetyl functionalities has been demonstrated[144]. The oligonucleotide reported here contains a hairpin stem-loop structure, which serves as lateral spacers between neighbouring oligodeoxynucleotides and as a linker arm between the glass surface and the single stranded sequence of interest. The main advantage of this method is that both 3′- and 5′-ends are not modified, thus making them favorable for various enzymatic and labeling reactions.

In a slightly different approach, a protocol using photochemical immobilization technique has been developed[83]. The method involves the covalent linking of the oligonucleotides to the surface during irradiation. Traditionally, psoralens, benzophenone, azides and carbenes are used for photochemical immobilization reactions, however, as these photoprobes suffer from several inherent drawbacks, anthraquinone has been employed, as in its excited state it can react with almost any C-H containing substrate. Using this conjugated system, oligonucleotides can be immobilized on surfaces such as polystyrene, polycarbonate, polypropylene, Teflon and silylated glass, etc.

As discussed above, a variety of surface chemistries have been developed for making synthetic oligonucleotide microarrays on solid- or polymeric surfaces[109,145]. The production and optimal performance of these arrays depends on some factors. One of them is a linker required to create a suitable distance between surface and the oligonucleotide sequence that is to be used for hybridization experiments; the distance minimizes the steric hindrances with the incoming molecules as well as provides accessibility to them. In some cases, polyethylene glycol[83] and oligothymidines[123] have been employed as spacers. Other factors include physical and chemical properties of surface[133,146], derivatization of slides with suitable functional groups, incorporation of suitable modified functional groups on oligonucleotides, density of oligonucleotides on the surface, delivery of tiny volumes of spotting solution, the blocking of

unreacted functional groups on the surface, length and type of target DNA molecules, hybridization and washing conditions, etc. Another problem related to uniform distribution of spotted oligonucleotide has been addressed by mixing a suitable solvent with properties, such as good wettability and low evaporation rate; betaine and dimethylsulfoxide are the most commonly used reagents for this purpose[147].

Conclusion

In the preceding discussion, various developments in the areas of cost-effective methods for oligonucleotide synthesis, preparation of oligonucleotide conjugates with biological and abiological ligands, and microarray (biochip) technology have been elaborated. From single stranded oligonucleotide to the double stranded siRNA, ribozymes and the peptide-nucleic acids — all are contributing towards building better prospects for oligonucleotides. Whether the need is diagnosis or treatment of a disease (cancer, viral diseases or inflammatory disorders to name a few)[148,149], the oligonucleotides will have a role to play. Further, a wide range of applications would emerge in diagnosis of diseases and therapeutics, as well as in unveiling many hitherto unknown functions of a wide range of nucleotide sequences.

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