1.1 Introduction

Nucleic acids are key molecules of life as they exist in every life form. These biomolecules, i.e., DNA and RNA, constitute the most important genetic material. DNA is conserved throughout the evolution of all living beings and is transmitted from the parents to their offspring. DNA, RNA and their products (proteins) work in a complicated yet organized fashion that eventually leads to the mystery of life. The stepping stone in understanding the structure and function of DNA in life processes was the discovery of hydrogen-bonded double helical structure of DNA by Watson and Crick\(^1\) in 1953 (Figure 1). Since then, the chemistry and biology behind nucleic acids have been of interest to many research groups all over the world. The post genomic era focuses mainly on the link between the DNA structure, the genes and the proteins they encode. The genetic information is encoded in the precise sequence of bases along each strand of DNA and flow of this information in the cells, controlled by DNA, consists of two basic steps, viz., transcription and translation.\(^2\) In the transcription step, DNA serves as a template for RNA synthesis, that in, turn acts as a template for the protein synthesis in the subsequent process of translation. After realizing the importance of DNA in life processes, scientists tried to synthesize DNA to explore the link between the structure and function of genes and the proteins. The functioning of specific genes and their role in human diseases can be understood with the help of molecular biology tools.

Oligonucleotide chemistry has been elemental to molecular biology and it can be said that synthetic oligonucleotides are the fuel that drive the engine of modern molecular biology. Nearly every technique, employed now a days in molecular biology, utilizes chemically synthesized oligonucleotides, which includes PCR, Real-Time PCR, antisense therapy, DNA sequencing, site directed mutagenesis, single-nucleotide polymorphism (SNP) assays, microarrays, and the rapidly expanding world of small RNAs.\(^3^\text{--}^8\)
In this day and age, synthetic oligonucleotides find applications in many biomedical fields. Small oligonucleotides are used in antisense therapy\(^4\) in which an oligonucleotide binds specifically to mRNA and inhibits translation. Some small oligonucleotide sequences called triplex forming oligonucleotides (TFO) can target the double stranded DNA,\(^9-16\) where they bind to the major groove of the DNA double helix by Hoogsteen base pairing. Catalytic RNAs and DNAs, called Ribozymes and DNAzymes, respectively, have been used for oligonucleotide-based therapeutics.\(^17,18\) Recently, short double stranded RNA duplexes, known as small interfering RNAs (siRNA), have been successfully used to silence gene function utilizing naturally occurring mechanism called RNA interference.\(^17,19\) Recently, short double stranded fragments of RNA, called microRNAs (miRNA), have shown potential for novel modulators of gene expression by targeting one or more mRNAs for translational repression or cleavage.\(^20,21\) Besides, synthetic oligonucleotides are finding their extensive use in diagnostics, sequencing and expression studies. In addition, synthetic oligonucleotides are also being used for the construction of microarrays which is a powerful tool for large-scale parallel analysis of genome sequences and gene expression in biological and biomedical research.\(^22-30\)
The following sections of this chapter will examine in greater detail the structure of oligonucleotides, the development of protocols for synthesis of oligonucleotides and their modified analogs, and the applications of these molecules in biomedical sciences.

1.2 Basic Constituents of Oligonucleotides

Oligonucleotides are short sequences (5 to 30 mer) of nucleotides linked via phosphodiester bonds. These structural units consist of three essential components: a nitrogenous base (a heterocyclic ring of carbon and nitrogen atoms), a pentose sugar (a five carbon sugar in heterocyclic ring form) and a phosphate group (Figure 2).

There are five types of bases in oligomers, viz., adenine (A) and guanine (G) are purine derivatives; cytosine (C), thymine (T) and uracil (U) are pyrimidine derivatives. Oligo-deoxyribonucleotides contain A, T, C and G bases attached with 2’-deoxyribose sugar, whereas oligo-ribonucleotides contain A, U, C and G bases attached to ribose sugar. The nitrogenous base is linked to position 1 of pentose sugar by an N-glycosidic bond through N⁰ of purines or N¹ of pyrimidines and such a unit is defined as a nucleoside. The phosphate esters of nucleosides are known as nucleotides.
1.3 Oligonucleotide Synthesis

The foremost published account of the chemical synthesis of an oligonucleotide was reported by Michelson and Todd\textsuperscript{31} in 1955. They synthesized a dithymidinyl nucleotide using phosphotriester chemistry. They prepared 3’-phosphoryl chloride of a 5’-benzoyl protected thymidine, using phenylphosphoryl dichloride. It was then reacted with 5’-hydroxyl of a 3’-protected thymidine. In this manner, they were able to link two thymidine nucleosides through a phosphate bond (Figure 3). Though the chemistry worked well, the phosphochloridate intermediate, formed during the reaction, was not stable, being susceptible to hydrolysis.

![Figure 3: Phosphoryl chloridate method by Michelson and Todd](image)

At around same time, Prof. H.G. Khorana along with his co-workers introduced a new concept of on-off protection and phosphodiester approach to oligonucleotide synthesis\textsuperscript{32-40} (Figure 4). Using the protocol, oligonucleotides of few bases could be synthesized conveniently. The method had advantages, as the efficiency of each coupling step can be calculated by measuring the absorbance of released DMTr cation.

![Figure 4: Phosphodiester approach by Khorana](image)
Moreover, Khorana et al. also introduced the protecting groups for nucleosidyl exocyclic amines that are even currently known as the standard protecting groups, isobutyryl for guanosine and benzoyl for adenosine and cytidine. Although, the method introduced the concepts of base protection and on-off chemistry for oligonucleotide synthesis, the utility of protocol was limited due to branching at the internucleotidic phosphate linkage and the time consuming purification steps required after each coupling step. Because of these limitations associated with the solution phase synthesis, Letsinger and his co-workers developed a method for polymer supported oligonucleotide synthesis using the phosphotriester method.\textsuperscript{41}

The major advantages of solid phase methodology over solution phase method were, viz., (i) the desired product always remained bound to the support throughout the chain elongation, (ii) large excess of reagents could be used in order to derive the reactions towards completion, (iii) the excess or unused reagents could be washed off easily without loss of products, (iv) allowed automation of methodology, and (v) no isolation or purification of intermediates was required. The protocol, developed by Letsinger et al.\textsuperscript{41}, was based on the concept introduced by Merrifield for peptide synthesis.\textsuperscript{42-44} Merrifield demonstrated that peptide synthesis reactions could be effectively carried out on an inert insoluble polymeric material by reversibly binding one of the reactants to the support, and after the reaction, the support-bound product could be easily released by appropriate cleavage conditions. Letsinger and his co-workers synthesized a dimer and a trimer oligonucleotides using the same polymeric support, a styrene-divinylbenzene support, however, it had an unfortunate property of swelling in some organic solvents and inhibited the rapid diffusion of the reagents and solvents through the matrix. Since then, considerable efforts have been invested towards the development of a suitable polymer support for the synthesis of oligonucleotides. Over the period of time, controlled pore glass (CPG), polystyrene and silica gel based solid supports have been developed for solid phase synthesis of oligonucleotides.

The introduction of trivalent phosphite compounds instead of pentavalent phosphate compounds, by Letsinger et al.\textsuperscript{45} in 1975, revolutionized the field of oligonucleotide synthesis. This approach, also known as
chlorophosphite method or phosphite-triester approach, was based on the fact that P^{III} derivatives are more reactive than corresponding P^{V} compounds. The major advantage of this chemistry was the significant reduction in time required for coupling due to the highly reactive nature of the nucleoside phosphomonochloridite intermediate. This approach not only made the oligonucleotide synthesis faster, but also opened a new window for the investigation of a variety of backbone modified oligonucleotides. However, the highly reactive nature of the nucleoside phosphomonochloridites made these intermediates very susceptible to hydrolysis. Moreover, the use of a highly reactive bifunctional phosphorodichloridite reagent inevitably leads to the formation of byproducts along with the desired one. Along with the formation of a desired 3'→5'- internucleotide linkage, two possible symmetrical products with 3'→3' and 5'→5' internucleotide linkages were also formed. Preparation of active reagent just before the coupling and maintenance of stringent conditions for coupling step, restricted the wide scale application of this methodology.

A significant breakthrough in synthesis chemistry was achieved by Serge Beaucage and Marvin Caruthers\textsuperscript{46} in 1981. They introduced phosphoramidite approach, in which phosphoramidite reagents were used that was prepared by replacing the reactive chloro group by various dialkylamines. Of these, diisopropyl substituted phosphoramidites were stable, non-hygroscopic, could be easily prepared and handled in open atmosphere. The use of phosphoramidite reagents permitted full and easy automation of oligonucleotide synthesis. In this approach, synthesis begins with the removal of 4,4'-dimethoxytrityl group from 5'-hydroxy with 1% TCA to obtain free hydroxyl groups at 5'-end of the nucleoside covalently bonded to solid support, the 5'-OH group subsequently reacts with the incoming nucleoside-phosphoramidite in the presence of an activator, tetrazole, to yield a dinucleoside-phosphate triester. The unreacted hydroxyls are capped with capping solution and oxidation of the triester with aqueous iodine solution generates stable dinucleoside phosphotriester. Thereafter, the cycle is repeated until the desired length of an oligonucleotide sequence is obtained. The oligomer is then cleaved from the solid support on treatment with concentrated...
ammonium hydroxide (30%) at 60°C for 16 h, which also removes the cyanoethyl groups from the internucleotidic phosphates and acyl protecting groups from exocyclic amino functions of the nucleic bases. The schematic diagram of this method is shown in figure 5.

Another strategy for oligonucleotide synthesis, **H-phosphonate approach**, was proposed almost simultaneously by two research groups, Garegg et al. and Froehler and Matteucci, in 1986. In this approach, 3′-H-phosphonate monomers were activated with an acyl chloride followed by reaction with 5′-hydroxyl of another nucleotide. The phosphonate linkages were stable and the approach offered an advantage that oxidation of the phosphonate linkages into phosphate linkages could be performed in a single step at the end of synthesis. However, H-phosphonate coupling reactions lead to a number of undesirable by-products. Therefore, this technique has not been as widely used for solid-phase oligonucleotide synthesis as phosphoramidite chemistry.
Successful development of stable phosphoramidite reagents, glass based non-permeable solid supports and automated DNA/RNA synthesizers have simplified the oligonucleotide synthesis, to an extent, that even a non-chemist can assemble these molecules quite easily without facing any problem.

1.4 Modified Oligonucleotides

The ease of customizing reaction cycles in automated solid-phase DNA synthesizers has allowed efficient and site-specific chemical modifications in oligonucleotides. Modified oligonucleotides are finding their use in a vast variety of biomedical applications. Modifications can be incorporated in synthetic oligonucleotides at nucleobases (purines or pyrimidines) / sugar / backbone and at the termini (3’- and/or 5’-end). The lower nucleophilicity of hydroxyl groups at 3’-and 5’-end of unmodified oligonucleotides limits the application of these biomolecules in biomedical studies, which generally require conjugation of these biomolecules with other moieties. Therefore, researchers have shown a preference for the synthesis of 3’- or 5’-end modified oligomers in comparison to alterations at nucleobases or sugar moiety, which involve laborious chemistry. Terminal modifications are easy to carry out and, moreover, they do not interfere in the duplex hybridization. End-modified oligonucleotides can be prepared either by using engineered polymer supports (for 3’-end modification) or by coupling the desired oligonucleotide chain with a modified phosphoramidite at the end of synthesis cycle (for 5’-end modification). In the following sections, some of the most commonly used terminal modifications have been described.

1.4.1 Amino-modified Oligonucleotides

(a) 3’-Amino modifications: Since the oligonucleotide synthesis proceeds from 3’- to 5’-direction, the methodology for 3’-end modifications involves the use of engineered polymer supports. A large number of such supports have been reported in literature for the generation of a free primary amino group at the 3’-terminus of oligonucleotide (Table-1).
Table 1: Supports for 3’-amination of oligonucleotides

<table>
<thead>
<tr>
<th>Support</th>
<th>Deprotection Conditions</th>
</tr>
</thead>
</table>
| ![Support](image1) | (1) 0.1 M DTT/MeOH, 30 min  
(2) NH₄OH, 60°C, 16 h |
| ![Support](image2) | Aq. NH₄OH, 60°C, 16 h |
| ![Support](image3) | Aq. NH₄OH, 60°C, 16 h |
| ![Support](image4) | Aq. NH₄OH, 60°C, 16 h |
| ![Support](image5) | Aq. NH₄OH, 60°C, 16 h |
| ![Support](image6) | Aq. NH₄OH, 60°C, 16 h |
| ![Support](image7) | Aq. NH₄OH, 60°C, 16 h |
| ![Support](image8) | (1) TBAF/THF, 30 min  
(2) NH₄OH, 60°C, 16 h  
(3) NaClO₄, 2 h  
(4) NH₂-(CH₂)₆-NH₂, NaCNBH₃ |

(a) 5’-Amino modifications: To introduce amino functionality at the 5’-end of oligonucleotides, modified β-cyanoethyl phosphoramidites are used at the end of synthesis cycle. A large number of amino-modifier phosphoramidites have been reported in literature for the introduction of a free primary amino group at the 5’-terminus of oligonucleotide. Some of the most commonly used 5’-amino-modifiers are depicted in table-2.
Table 2: Reagents for 5’-amination of oligonucleotides

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Deprotection Conditions</th>
</tr>
</thead>
</table>
| \[
\text{N}-\text{CH}_2\text{CH}_2\text{O}-\text{P} \quad \text{O(\text{CH}_2)_n\text{NHCOCF}_3} \quad n = 2,6
\] | Aq. NH$_4$OH, 60ºC, 16 h |
| \[
\text{N}-\text{CH}_2\text{CH}_2\text{O}-\text{P} \quad \text{O(\text{CH}_2)_n\text{NHDAtm}} \quad n = 3,5,6
\] | Aq. NH$_4$OH, 60ºC, 16 h |
| \[
\text{N}-\text{CH}_2\text{CH}_2\text{O}-\text{P} \quad \text{O(\text{CH}_2\text{CH}_2\text{O})_3\text{CH}_2\text{CH}_2} \quad \text{O} \quad \text{N} \quad \text{O}
\] | Aq. NH$_4$OH, 60ºC, 16 h |
| \[
\text{N}-\text{CH}_2\text{CH}_2\text{O}-\text{P} \quad \text{O} \quad \text{NHCOCF}_3 \quad \text{Base}
\] | Aq. NH$_4$OH, 60ºC, 16 h |
| \[
\text{N}-\text{CH}_2\text{CH}_2\text{O}-\text{P} \quad \text{O} \quad \text{NHfomoc} \quad \text{Base}
\] | Aq. NH$_4$OH, 60ºC, 16 h |
| \[
\text{N}-\text{CH}_2\text{CH}_2\text{O}-\text{P} \quad \text{O} \quad \text{U} \quad \text{BzO} \quad \text{OBz}
\] | (1) Aq. NH$_4$OH, 60ºC, 16 h  
(2) NaClO$_3$, 2 h  
(3) NH$_2$(CH$_2$)$_n$-NH$_2$, NaCNBH$_3$ |

1.4.2 Thiol-modified Oligonucleotides

(a) 3'-Thiol modifiers: 3’-Thiol modified oligonucleotides are used in a variety of coupling reactions and can be easily synthesized by using linkers
with disulphide bonds. Table-3 shows some of the supports for introducing mercaptoalkyl functionality at the 3'-end of oligonucleotides.

**Table-3: Supports for 3'-thiolation of oligonucleotides**

<table>
<thead>
<tr>
<th>Support</th>
<th>Deprotection Conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>O(CH_2_2-S-S-(CH_2)n-ODMTr _ n = 3, 6</td>
<td>(1) 0.1 M DTT/MeOH, 30 min (2) Aq. NH_4OH, 60ºC, 16 h</td>
</tr>
<tr>
<td>O(CH_2_2-S-S-(CH_2CH_2O)_2CH_2CH_2ODMTr</td>
<td>(1) 0.1 M DTT/MeOH, 30 min (2) Aq. NH_4OH, 60ºC, 16 h</td>
</tr>
<tr>
<td>NHCO(CH_2_2-S-S-(CH_2)_2CONH(CH_2)_2ODMTr</td>
<td>(1) 0.1 M DTT/MeOH, 30 min (2) Aq. NH_4OH, 60ºC, 16 h</td>
</tr>
</tbody>
</table>

(b) *5’-Thiol modifiers:* 5'-Thiol modified oligonucleotides can be easily synthesized by employing mercaptoalkylated phosphoramidites. In addition to these methods, thiol group can also be introduced at 5'-end by indirect methods i.e., by reacting 5'-aminoalkylated oligonucleotides dithiobis-N-(succinimidyl)propionate (DSP), followed by DTT treatment to yield the desired 5'-thiolated oligomers. Table 4 reports some of the reagents used for 5'-thiolation of oligonucleotides.

**Table 4: Reagents for 5'-thiolation of oligonucleotides**

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Deprotection Conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>CH_3O-P-_O(CH_2)_n-Str _ n = 3,6</td>
<td>Aq. NH_4OH, 60ºC, 16 h</td>
</tr>
<tr>
<td>CH_3O-P-_O(CH_2)_n-Str</td>
<td>Aq. NH_4OH, 60ºC, 16 h</td>
</tr>
<tr>
<td>NC-CH_3CH_2O-P-_O(CH_2)_n-Str _ n = 3,6</td>
<td>Aq. NH_4OH, 60ºC, 16 h</td>
</tr>
</tbody>
</table>
1.4.3 Phosphate-modified Oligonucleotides

(a) 3’-Phosphate modifiers: Phosphorylation at 3’-end of oligonucleotides can be achieved in several ways following solid phase or solution phase methods. Markiewicz et al.\textsuperscript{48} in 1989, reported universal solid supports for the synthesis of oligonucleotides with terminal 3’-phosphate. Briefly, two types of solid supports were prepared. CPG was modified with: (i) methacrylic acid derivatives and 2-mercaptoethanol or (ii) aminoalkylsilane, succinic anhydride and benzidine. These supports are compatible with established phosphoramidite chemistry of oligonucleotides synthesis, giving rise to an oligonucleotide with terminal 3’-phosphate function during final deprotection. Table-5 reports some of the supports listed in literature for the synthesis of 3’-phosphorylated oligonucleotides.

Table 5: Supports for 3’-phosphorylation of oligonucleotides

<table>
<thead>
<tr>
<th>Support</th>
<th>Deprotection Conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image1.png" alt="Support 1" /></td>
<td>(1) 0.1 M DTT/MeOH, 30 min, rt</td>
</tr>
<tr>
<td></td>
<td>(2) Aq. NH₄OH, 60°C, 16 h</td>
</tr>
<tr>
<td><img src="image2.png" alt="Support 2" /></td>
<td>(1) 0.1 M DTT/MeOH, 30 min, rt</td>
</tr>
<tr>
<td></td>
<td>(2) Aq. NH₄OH, 60°C, 16 h</td>
</tr>
<tr>
<td><img src="image3.png" alt="Support 3" /></td>
<td>Aq. NH₄OH, 60°C, 16 h</td>
</tr>
<tr>
<td><img src="image4.png" alt="Support 4" /></td>
<td>(1) AcOH, 4 h</td>
</tr>
<tr>
<td></td>
<td>(2) Aq. NH₄OH, 60°C, 16 h</td>
</tr>
<tr>
<td><img src="image5.png" alt="Support 5" /></td>
<td>(1) AcOH, 4 h</td>
</tr>
<tr>
<td></td>
<td>(2) Aq. NH₄OH, 60°C, 16 h</td>
</tr>
</tbody>
</table>
(a) 5'-Phosphate modifiers: In literature, a number of reagents have been proposed for the preparation of 5'-phosphorylated oligonucleotides. A phosphoramidite-based reagent, (2-cyanoethoxy)-2-(2′-O-4,4′-dimethoxytrityloxyethylsulfonyl)-ethoxy-\(N,N\)-diisopropylaminophosphine, has been reported for the 5'-phosphorylation of oligonucleotides. Table 6 summarizes some of the reagents and their deprotection conditions.

Table 6: Reagents for 5'-phosphorylation of oligonucleotides

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Deprotection Conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image1.png" alt="Chemical Structure 1" /></td>
<td>NH(_4)OH, 60°C, 16 h</td>
</tr>
<tr>
<td><img src="image2.png" alt="Chemical Structure 2" /></td>
<td>NH(_4)OH, 60°C, 16 h</td>
</tr>
<tr>
<td><img src="image3.png" alt="Chemical Structure 3" /></td>
<td>NH(_4)OH, 60°C, 16 h</td>
</tr>
<tr>
<td><img src="image4.png" alt="Chemical Structure 4" /></td>
<td>NH(_4)OH, 60°C, 16 h</td>
</tr>
<tr>
<td><img src="image5.png" alt="Chemical Structure 5" /></td>
<td>NH(_4)OH, 60°C, 16 h</td>
</tr>
<tr>
<td><img src="image6.png" alt="Chemical Structure 6" /></td>
<td>NH(_4)OH, 60°C, 16 h</td>
</tr>
</tbody>
</table>
1.4.4 Other Modifications

(I) **Hydrazide-modified oligonucleotides:** There are only a few examples in the literature, which utilize hydrazide modified oligonucleotides.\(^49\)\(^-\)\(^51\) In all the cases, the hydrazide functionality was introduced post-synthetically. Schweitzer and his co-workers\(^50\) have described an efficient synthetic approach to produce tailored hydrazide modified oligonucleotides for solution phase conjugation and surface attachment applications.

(II) **Aminooxy-modified Oligonucleotides:** Oligonucleotides containing aminooxy functionalities find numerous applications as they offer an advantage that aminooxy oligomers can specifically be derivatized in the presence of aminoalkylated oligomers.\(^52\)\(^-\)\(^54\). The terminal modified oligonucleotides have been synthesized by using 
\[
O-(11-Phthalimidoxy-3,6,9-trioxaundecyl)-O-(2-cyanoethyl)-(N,N-diisopropyl)phosphoramidite,\]
which was coupled to the desired oligonucleotide sequence at the 5’-end. The modified oligonucleotides were subsequently deprotected by 0.5 M hydrazine hydrate in Py/AcOH (4:1 v/v) for 30 min followed by aqueous ammonium hydroxide for 8 h at 60°C.

(III) **Phosphorothioate Oligonucleotides:** The synthesis of phosphorothioate oligonucleotides is carried out in a manner that is very similar to that of unmodified oligonucleotides by the use of phosphotriester, phosphoramidite and \(H\)-phosphonate methods. One of the non-bridging oxygens is exchanged with sulfur in a conservative manner, as the negative charge of the phosphate group is retained. To prepare these oligomers, the oxidation step comprising of aqueous iodine treatment is replaced by suitable sulfurizing reagents.

Table-7 summarizes some of the supports and reagents reported for end modification of oligonucleotides along with their deprotection conditions.
Table-7: Supports and reagents for end modifications and their deprotection conditions

<table>
<thead>
<tr>
<th>Modification</th>
<th>Support / Reagent</th>
<th>Deprotection Conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>5’-azido</td>
<td><img src="image" alt="5’-azido" /></td>
<td>Aq. NH₄OH, 60°C, 16h</td>
</tr>
<tr>
<td>5’-diene</td>
<td><img src="image" alt="5’-diene" /></td>
<td>Aq. NH₄OH, 60°C, 16h</td>
</tr>
<tr>
<td>3’-carboxy</td>
<td><img src="image" alt="3’-carboxy" /></td>
<td>(1) Pd₂(dba)₃, CHCl₃&lt;br&gt;(2) n-Butylamine in formic acid&lt;br&gt;(3) NH₄OH, 60°C, 16 h</td>
</tr>
<tr>
<td>5’-aminoxy</td>
<td><img src="image" alt="5’-aminoxy" /></td>
<td>(1) 0.5 M Hydrazine hydrate in Py/H₂O&lt;br&gt;(2) Aq. NH₄OH, 60°C, 8 h</td>
</tr>
<tr>
<td>5’-hydrazone</td>
<td><img src="image" alt="5’-hydrazone" /></td>
<td>(1) Aq. NH₄OH, 60°C, 2 h&lt;br&gt;(2) 80% AcOH, 30min</td>
</tr>
<tr>
<td>5’-hydrazone</td>
<td><img src="image" alt="5’-hydrazone" /></td>
<td>(1) Aq. NH₄OH, 60°C, 2 h&lt;br&gt;(2) 80% AcOH, 30min</td>
</tr>
<tr>
<td>5’-α-oxo aldehyde</td>
<td><img src="image" alt="5’-α-oxo aldehyde" /></td>
<td>(1) 0.4M NaOH in MeOH/H₂O containing DTT at room temp for 17 h</td>
</tr>
</tbody>
</table>
1.5 Applications of Synthetic Modified Oligonucleotides

In the modern years, modified oligonucleotides have become an indispensable tool for various applications in modern molecular biology. These biomolecules find extensive use for therapeutic and diagnostic applications in biomedical research. They have been used for assignment of gene function, detection of genetic diseases, elucidation of enzyme mechanisms, fluorescent microscopy, hybridization affinity chromatography, sequencing etc. Short oligonucleotide sequences are used in antisense and antigene therapies for the inhibition of gene expression. Besides therapeutics, recently, modified oligomers are widely being used in the fabrication of microarrays which is a prevailing tool for gene expression studies, sequencing and disease diagnosis.

1.6 Oligonucleotide Microarrays

Over the past few years, microarray technology has emerged as a powerful tool for large-scale parallel analysis of genome sequences and gene expression in biological and biomedical research, and currently represents an extensive, maturing scientific method that accompany commercial industry. The basic principle of microarray technology is same as the conventional hybridization techniques (e.g. Northern blot). The hybridization assay relies on the ability of a single-stranded nucleic acid to bind a complementary molecule and form a stable duplex. The base-pairing property gives single strand the ability to recognize its complementary strand to form a duplex. Oligonucleotides or DNA molecules with known sequences are attached at pre-selected positions on a solid polymeric support which is put in contact with solution containing the targets to be detected. The surface immobilized single stranded probes bind to their labeled complements in solution to detect and quantify targets. The microarray is then scanned or imaged to obtain the complete hybridization pattern.

DNA arrays are topical owing to their promising ability for obtaining information on nucleic acid sequences faster, simpler and cheaper than traditional methods. The major applications of this technique include, large scale screening of mutations, studies of gene polymorphism, gene expression
analysis and disease diagnosis. The development of microarray technology has been the result of the assimilation of many different disciplines such as molecular biology, genetics, advanced micro-fabrication and micromachining technologies, nucleic acid chemistry, surface chemistry, analytical chemistry, software and robotics, and automation. Microarray technology represents a truly successful synergy of these many different scientific and engineering fields.

There are two formats of DNA microarrays in terms of microarray contents, namely, (i) Oligonucleotide microarrays, and (ii) cDNA microarrays. In oligonucleotide microarray format, synthetic oligonucleotides (usually 20 to 80mer) are arrayed on solid polymeric supports either by on-chip synthesis or by conventional synthesis, followed by immobilization on the platform. cDNA microarrays contain long DNA sequences derived from cDNA or PCR products immobilized on a solid substrate. In a cDNA microarray format, customized cDNA probes are immobilized using robotics-mediated spotting. The DNA fragments spotted can be PCR fragments amplified from specific target sequences, or they can be library clones.

Though the concept of microarray for nucleic acid analysis originated from oligonucleotides, cDNA microarrays were rapidly acknowledged as they are simpler to prepare and the DNA sequences are available in many biochemical laboratories. cDNA microarrays, however suffer from low reproducibility and cross-hybridization with undesirable target DNA. Consequently, oligonucleotide microarrays have gained tremendous popularity in recent years. Oligonucleotide microarrays are highly specific in sequence specificity and flexible to allow variation in probe lengths.

1.6.1 Fabrication of Oligonucleotide Microarrays

For the construction of oligonucleotide microarrays, two approaches have emerged. The first approach uses the direct spotwise synthesis of oligonucleotides on a chip surface (in-situ synthesis). In this method, molecules are added in a sequential manner, i.e., one monomer is added to the previous one, with the consequence that each array is made as an original with a reduced throughput. The second method relies on immobilization of previously
synthesized oligonucleotides by spotting on functionalized surfaces (deposition method). The following sections will explain the methodologies.

1.6.1.1 *In-situ or on-chip Synthesis of Oligonucleotide Microarrays*

The *in-situ* process involves synthesis of oligonucleotides directly on the solid polymeric surfaces at the pre-determined sites. In literature, a number of protocols for *in-situ* synthesis of oligonucleotide microarrays have been reported. Southern et al. were the first to demonstrate the use of glass plates as substrates for *in-situ* synthesis of oligonucleotide arrays using standard phosphoramidite chemistry. The arrays, constructed by this method, were directly used for hybridization assays following usual deprotection. Later, Matson and co-workers in collaboration with southern’s group explored the polypropylene films as a substrate for array synthesis. Polypropylene was a substrate of choice owing to its inertness to most chemical reagents used during oligonucleotide synthesis. Moreover, it offers advantages such as negligible fluorescent background, low non-specific adsorption of proteins and nucleic acids, easy to derivatize and inexpensive. Using the photolithographic technique, Fodor et al. have revealed the mask directed *in-situ* synthesis (figure 6), which is still considered to be the most successful one, using phosphoramidites protected with a photolabile group, α-methyl-6-nitropiperonyloxycarbonyl, for creating high density arrays on silica substrates.

![Figure 6: In situ fabrication of oligonucleotide microarrays using photolithographic technique.](image-url)
This fundamental technology involves the use of photolithographic masks, to achieve selective light exposure at pre-determined area on the solid surface. After the coupling, the photo-labile groups are cleaved from the terminus of the growing chains by illuminating the selected surface area to produce a pattern of free hydroxyl groups while in non-illuminated surface area, the hydroxyl groups remain protected. The reaction surface is subsequently exposed to a solution containing protected-nucleoside phosphoramidite monomer. To couple with the free hydroxyls to extend the chains by one nucleotide, the photo-masking, illumination, and the coupling steps are repeated with each cycle, adding a different nucleotide monomer according to the sequences to be synthesized. Recently, Philips et al.\textsuperscript{60} have described a method for \textit{in-situ} synthesis oligonucleotide microarrays on carbon-based materials using light-directed photolithographic phosphoramidite chemistry. The process permits the synthesis of high density oligonucleotide chips. However, low reaction efficiency leading to accumulation of short sequences, lack of flexibility and use of expensive photomasks equipment limit the suitability of the protocol for generation of cost-effective microarrays.

1.6.1.2 Deposition method for Microarray Fabrication

The deposition or spotting method is the most widely used tool employed by research laboratories for routine applications for creating low-to-medium density microarrays. This method depends on the post-synthetic immobilization of oligonucleotides on to solid polymeric supports. Using this method, microarrays are constructed in three basic steps, viz., (i) the oligonucleotides are synthesized on an automated gene-synthesizer and deprotected using the standard conditions, (ii) desalted oligonucleotides are dissolved in appropriate buffers and spotted onto a substrate of choice, and (iii) after completion of reaction, the microarrays are washed and dried. Subsequently, these constructed microarrays are subjected to hybridization assay followed by visualization under a laser scanner and data analysis.

This method offers advantages over \textit{in-situ} method, in terms of choice of substrate, biomolecules to be attached, and chemistry employed. The quality of
microarrays, constructed by deposition method, depends considerably on the substrate employed and the chemistry used for attachment of oligonucleotides.

1.6.1.2.1 Substrates for Microarray Construction

Various polymeric and solid supports have been used as substrates for immobilization of oligonucleotides. The suitability of a support depends on its storage stability and ease of functionalization. In literature, a myriad of substrates such as glass, polypropylene, modified silicon surfaces, gold surfaces, optical fibers, carbon layer, polyacrylamide gel pads etc., have been proposed for construction of biochips. However, glass remains a surface of choice for microarray applications, owing to its easy availability, low intrinsic fluorescence, excellent flatness, chemical durability, and low cost.

Besides glass, Fixe et al. have reported a method for array fabrication of biomolecules using modified polymethymethacrylate (PMMA) surfaces. In another efficient approach, Consolandi et al. have described a method for the construction of oligonucleotide microarrays using glass microslides coated with an amine-rich polysaccharide, chitosan.

Recently, another polymer, SU8, has gained popularity as a support coating for immobilization of biomolecules. SU-8 is an epoxy-based negative photoresist with strong hydrophobic character. Being economically viable material, it has been used to create layers sizes from 30nm up to a few microns. Its thermal and chemical stability as well as light transparency above 360 nm make SU-8 ideal for microarray fabrication. Recently, Erkan et al. have demonstrated a method for non-covalent immobilization of cholesteryl-TEG-modified oligonucleotides on SU-8 surfaces. High epoxy-group density makes SU8, an excellent support for microarray fabrication.

In addition to organic coatings, a number of inorganic polymer coatings, such as carbon layer, oligomeric silsesquioxane, silicon wafers etc., have also been proposed for array preparation. Thin gold layers have been used for the direct immobilization of thiol modified oligomers. The natural adsorption of organosulfur compounds on gold is a widely used method for the preparation of self-assembled monolayers (SAMs).
Lately, carbon based supports have gained popularity as substrates for microarray fabrication. Amorphous carbon thin films are particularly attractive substrates, as they can be reproducibly fabricated at room temperature offering the ability to amalgamate the chemical stability of carbon with substrates that are not amenable to array fabrication. Recently, Lockett et al.\textsuperscript{96} have reported an aldehyde-terminated amorphous carbon-based substrate for the immobilization of amine-modified oligonucleotides. Oligonucleotide arrays prepared by this method showed a signal-to-noise ratio 40% higher than those prepared on gold SAMs. This can be attributed to lower background fluorescence intensity and brighter oligonucleotide features on the amorphous carbon substrate as compared to gold SAMs.

### 1.6.1.2.2 Strategies for Immobilization of Oligonucleotides

In the deposition method, the immobilization of pre-synthesized oligonucleotides onto the substrate can be achieved in two ways:

(A) Immobilization via non-covalent interactions

(B) Immobilization via covalent linkage

(A) Immobilization via Non-covalent Interactions

In early stages of array development, the non-covalent immobilization strategy was the most commonly used method. The first non-covalent attachment approach was developed by Wilchek and his co-workers,\textsuperscript{97} which was based on interaction of biotin with streptavidin or other avidin species. The surface bound streptavidin molecules can easily immobilize biotin molecules due to formation of a stable complex. Other method of non-covalent immobilization involves binding of negatively charged phosphodiester groups of oligonucleotides with the positively charged amino functions of surface-bound poly-L-lysine side-chains by electrostatic forces.

In addition to this, Belosludtsev et al.\textsuperscript{98} have described a straightforward and reproducible technique for the production of arrays, employing non-covalent attachment of unmodified oligonucleotide probes to an amino-silanized glass surface. The advantage of the method is that the affinity and selectivity of the duplex formation are achieved as good as can be obtained by traditional
methods of immobilization. Further, the adsorption-based methods require relatively very low concentration of unmodified probes.

Non-covalent immobilization protocols are easy to carry out, however, these are not very appropriate for construction of arrays. Microarrays, constructed by biotin-streptavidin chemistry, were susceptible to desorption under the conditions used for hybridization. The charge based immobilization also had several drawbacks. Due to multiple-point attachment of the probe with the positively charged surfaces, the probe lies flat on the surface that leads to poor hybridization efficiency and low signal to noise ratio. Owing to these limitations, covalent immobilization has been preferred to selectively link the probes on to a surface of choice.

(B) Immobilization via Covalent Interactions

Covalent immobilization of short oligonucleotides is the preferred approach for the construction of oligonucleotide microarrays. The terminal covalent attachment allows the entire oligonucleotide to be available for hybridization and to withstand the high temperature and salt concentrations, often required during the rigorous washing conditions in subsequent steps of processing. Methods reported for covalent attachment of oligonucleotides generally involve the use of pre-activated solid supports, homo- or heterobifunctional cross-linkers/reagents and modified oligonucleotides. In general, the immobilization of oligonucleotides via covalent bonding can be achieved in two ways:

(i) Covalent attachment under the influence of light (photochemical methods)
(ii) Covalent attachment via activation of the substrate functionality and/or the oligonucleotide (thermochemical methods)

(i) Photochemical Methods

Covalent ligand immobilization via photoreactive species (photoprobes), i.e. the covalent linkage to the surface is mediated by the photoprobe during irradiation, offers the enthralling possibility of selective activation. In principle, the method offers a specific one-step procedure that is applicable for ligand immobilization on a wide range of surfaces. Conventionally, benzophenones, psoralens, and precursors of nitrenes, and carbenes have been used in
photochemical immobilization reactions. However, these groups suffered from several drawbacks. Carbenes and nitrenes are highly electrophilic species that react readily with reaction solvents thus significantly reducing the immobilization yields. Psoralens react only with solid surfaces that contain exposed double bonds. Benzophenones react photochemically in radical-like reactions and do not interact with the reaction solvents, however, due to nearly overlapping absorption spectra of benzophenones with the biological materials, there is an increased risk of photoinduced damages. Furthermore, the thermal reactivity of ketones increases the risk of side reactions between the ketones and multifunctional bio-ligands in the aqueous medium.

As these photoprobes have several limitations, in the recent years, anthraquinone has gained much attention as a photoprobe. Anthraquinone has a unique property of reacting with substrates containing C-H bonds. Koch et al. performed photochemical immobilization of oligonucleotides using anthraquinone as a photoprobe. Anthraquinone-phosphoramidite reagents were employed for the synthesis of oligonucleotides with anthraquinone at 5'-terminus. The method has presented a facile, orientation-specific, one step technique for photochemical immobilization of oligomers using anthraquinone on a variety of surfaces such as polystyrene, polycarbonate, polypropylene, teflon and modified glass, etc.

To simplify this approach, a new heterobifunctional reagent, N-(3-trifluoroethanesulfonyloxypropyl)-anthraquinone-2-carboxamide (NTPAC), was developed by Kumar et al. for the photochemical immobilization of biomolecules on a variety of surfaces. The trifluoroethanesulfonate ester group of the reagent reacts with aminoalkyl- or mercaptoalkyl- functions present in the biomolecules, and the anthraquinone moiety reacts with a variety of carbon-containing polymers under ultraviolet irradiation (Figure 7).

The reagent, NTPAC, was used in two ways, viz., (i) the reagent was first brought in contact with the modified glass slide and exposed to long wavelength ultraviolet light (365 nm), thereby generating active trifluoroethanesulfonate ester functions on the support, which subsequently reacted with mercaptoalkyl- or aminoalkyl-modified oligonucleotides to fix them on the support, and (ii) the
reagent was allowed to react first with 3'- or 5'-aminoalkyl- or mercaptoalkyl-modified oligonucleotides to form the oligonucleotide-anthraquinone conjugate, which was then brought in contact with a modified glass surface and exposed to long wavelength ultraviolet light (365 nm), resulting in immobilization of the conjugate on the support.

Both the routes worked satisfactorily and the constructed microarrays showed high thermal and pH stability. The constructed microarrays were also tested for hybridization assay. However, the use of an expensive and a highly moisture sensitive reagent, trifluoroethanesulfonyl chloride, limits its widespread application.
On the parallel lines, recently, Patnaik et al.\textsuperscript{109} reported the synthesis and application of a new heterobifunctional reagent, N-(iodoacetyl)-N'-(anthraquinon-2-oyl)-ethylenediamine (IAED) for immobilization of modified oligonucleotides via two different routes (Figure 8).

The iodoacetyl group provides specificity and reactivity towards mercaptoalkyl- and thiophosphoryl-functionalities. Owing to this specificity, mercaptoalkyl- and thiophosphorylated oligonucleotides were immobilized on the modified glass microslides under the influence of UV light and microwaves. The thermochemical reaction between mercaptoalkyl group and iodoacetyl functionality was carried out under microwaves and the photochemical reaction involving anthraquinone and modified glass slide was performed under UV light.

![Figure 8: Preparation of the reagent, IAED, and immobilization of modified oligonucleotides onto virgin glass surface](image)
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The reaction between thiolated oligomers and the iodoacetyl moiety of the reagent was found to be much faster under microwaves (MW) (10 min) as compared to 2 h and 45 min under thermal conditions (45°C). The immobilized probes were found to be thermally stable and the quality of the constructed oligonucleotide microarrays was tested by performing a hybridization assay with a complementary labeled target. Subsequently, the fabricated microarrays were used for the detection of base mismatches.

More recently, another heterobifunctional reagent, 1-N-(maleimidohexanoyl)-6-N-(anthraquinon-2-oyl) hexanediamine (MHAHD) possessing a photoactive anthraquinone moiety on one terminus and an electrophilic maleimide group on the other one, has been demonstrated to affect efficient immobilization of thiolated oligonucleotides on a modified glass surface (Figure 9). The addition of thiolated oligonucleotides to maleimidoalkyl group was accelerated by performing reaction under microwaves.

Such reactions are generally fast and clean, since they avoid post-immobilization processing. In another approach, Kimura et al. 111,112 employed a
different strategy that permitted the immobilization of non-modified probes on a poly-carbodiimide coated glass surface by UV irradiation. The methodology offers an economical and rapid route for preparation of biochips as compared to the existing protocols.

In an alternate approach, Preininger et al.\textsuperscript{113} have used photoactivatable copolymers of vinylbenzylthiocyanate as immobilization matrix for biochips. In this method, the polymer, poly(styrene-co-4-vinylbenzylthiocyanate), on exposure to UV light ($\lambda = 254$ nm), transforms into the corresponding isothiocyanate, which reacts with amino-modified oligonucleotides forming a stable thiourea linkage. The photogenerated isothiocyanate units were employed for improved adhesion of the reactive layer of the polymer to the substrate as well as for covalent immobilization of amino-terminated biomolecules.

Recently, Gudnason et al.\textsuperscript{114} has described a very simple, and inexpensive method for the construction of biochips on unmodified glass slides. A 14-base oligonucleotide with a poly(T)10 – poly(C) 10 tails (TC tag) was linked to the glass slide by UV light irradiation. Probes immobilized onto unmodified glass microscope slides performed similarly to probes bound to commercial amino-silane–coated slides and had comparable detection limits. The prepared arrays were thermally stable as constructed biochips did not showed any significant decrease in hybridization performance after a 20 min incubation in water at 100°C prior to rehybridization.

Another elegant approach, for the photochemical immobilization of oligomers on glass slides, has been proposed by Schuler et al.\textsuperscript{115} The immobilization of single-stranded capture oligonucleotides was accomplished by UV cross-linking on silanated (amino and epoxy), and unmodified glass surfaces. Unmodified single-stranded DNA molecules can be directly immobilized onto amino- and epoxy surfaces by UV cross-linking.

(ii) \textbf{Thermochemical Methods}

In this strategy, reactive functional groups are introduced in the oligomers during conventional automated synthesis that can form covalent bonds under thermal conditions with the complementary groups on the surface in the presence/absence of a suitable coupling or condensing agents.
Oligonucleotides containing linkers at the internal positions or at the termini (3’- or 5’-end) are most commonly employed in this method. These oligomers are synthesized separately, purified and are then immobilized at defined sites on a solid surface. An important aspect of this method is single point attachment of oligonucleotides that allows the entire molecule to be accessible for the hybridization. Also, the covalent linkage provides stability, which is required to withstand stringent washing conditions used in microarray processing. In addition, the method allows flexibility in terms of biomolecules to be attached and the chemistry employed. A vast number of biomolecules, viz., oligonucleotides, peptides, PNA, proteins and so forth, can be covalently fixed up on the chemically modified/ or unmodified surfaces.

In the past few years, some of the well studied methods include the activation of the substrate with standard homo- or heterobifunctional reagents. One of the most commonly employed homobifunctional reagents is phenylene diisothiocyanate (PDITC) for the immobilization of amino- or thiol-modified oligomers. In 2001, Sheng et al.\textsuperscript{116} compared three different immobilization strategies employing three different homobifuntional reagents, viz., glutaraldehyde, succinic anhydride and phenylene diisothiocyanate (PDITC), to attach aminoalkylated oligomers onto glass surface. The immobilization efficiencies turned out to be $2.55 \times 10^{13}$ probes/cm$^2$ for glutaraldehyde, $3.21 \times 10^{13}$ probes/cm$^2$ for succinic anhydride and $6.68 \times 10^{13}$ probes/cm$^2$ for PDITC reagent. The results indicated that highest immobilization efficiency was obtained using PDITC.

In addition to homobifunctional reagents, several heterobifunctional have also been reported in literature for the fabrication of microarrays.\textsuperscript{116-122} Some of the reagents that have been used for the immobilization of amino- and mercaptoalkylated oligomers are succinimidyl 4-[maleimidophenyl] butyrate (SMPB), $m$-maleimidobenzoyl-$N$-hydroxysuccinimide ester (MBS), succinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate (SMCC), $N$-(g-maleimidobutryloxy) succinimide ester (GMBS), $m$-maleimidopropionic acid-$N$-hydroxysuccinimide ester (MPS) and $N$-succinimidyl(4-idoacetyl) aminobenzoate (SIAB).
In 2004, Kumar et al.\textsuperscript{120} reported an elegant approach for the immobilization of amino- and mercaptoalkylated oligonucleotides using a novel heterobifunctional reagent, \textit{N-}(2-trifluoroethanesulfonatoethyl)-\textit{N-}(methyl)-triethoxysilylpropyl-3-amine (NTMTA). The microarrays were fabricated via two routes (Figure 10). In the first route, 5'-amino- or mercaptoalkylated oligonucleotides were allowed to react with NTMTA to form an oligonucleotide-triethoxysilyl conjugate, which, in a subsequent step, on reaction with virgin glass surface, results in surface-bound oligonucleotides. In the second route, the NTMTA reagent reacts first with a glass microslide generating trifluoroethanesulfonate ester functionalities, which react with 5'-amino- or mercaptoalkylated oligomers to produce oligonucleotide microarrays. The fabricated microarrays were successfully used in single and multiple nucleotide mismatch detection.

Figure 10: Immobilization of amino- and mercaptoalkylated oligonucleotides using NTMTA

In another approach, Choithani et al.\textsuperscript{121} have reported the synthesis of a glass-specific heterobifunctional reagent, \textit{N-}(3-triethoxysilylpropyl)-6-(\textit{N}-...
maleimido)-hexanamide (TPMH), for the immobilization of 3'- or 5'-mercaptoalkylated oligomers under the influence of microwaves (Figure 11). The triethoxysilyl group is specific toward virgin glass surface and maleimide function undergoes conjugate addition to 3'- or 5'-mercaptoalkyl-modified oligonucleotides. Immobilization of oligonucleotides has been established via two routes, viz., as discussed above. The fabricated chips were successfully employed in detection of match/mismatches in the targets based on variation in the fluorescence intensity. The perfect matched duplex gave the highest fluorescence intensity, while the single and double mismatched duplexes exhibited fluorescence in decreasing order.

![Figure 11: Immobilization of mercaptoalkylated oligonucleotides using TPMH](image)

Very recently, Misra et al.,\textsuperscript{122} have reported a new heterobifunctional reagent, N-(3-triethoxysilylpropyl)-4-(isothiocyanatomethyl)-cyclohexane-1-carboxamide (TPICC), for the immobilization of self-quenched hairpin probes on glass surface for sensitive detection of oligonucleotides. Fluorescence of the fluorophore was quenched in the closed state, due to the presence of guanosine
residues in close proximity of fluorophore. On hybridization, the duplex unwinds leading to restoration of fluorescence of the fluorophore. Efficiency, specificity and thermal stability at variable temperature and pH conditions were studied for the constructed microarrays. Figure 12 shows the schematic representation of the strategy.

Two protocols comprise of surface activation with maleimide providing activated double bonds that are reactive towards thiolated oligonucleotides and the substitution of a thiolated substrate with an acrylamide-capped oligomers (or vice versa) have also been reported. Other elegant options include the use of combinations such as, organic-inorganic interactions involving thiolated oligonucleotides on gold surface, coordination complexes employing phosphorylated oligonucleotides on zirconylated-surface and so forth.

![Figure 12: Immobilization of hairpin probes onto virgin glass surface using TPICC](image-url)
Lane et al.\textsuperscript{125} revealed that oligonucleotide probes bearing a poly (dG) spacer immobilized on a zirconium phosphonate surface, led to a higher target capture, compared to probes with either no spacer or a different polynucleotide (polyA, polyC and polyT) spacer, during hybridization with complementary targets. The higher probe coverage, due to formation of G-quadruplexes, resulted in improved affinity of the probes for zirconium surface.

Activation strategies involving coupling or condensing reagents have also been used for covalent binding of probes to the surface. However, these strategies often result in the irreproducible and unreliable immobilization performance. A simple and direct chemistry that does not require additional activation/condensing reagent is always preferred. Therefore, the chemistry used for immobilizing oligonucleotides is derived, in many cases, from rather straightforward organic reactions. In one of the previous studies, epoxy functions were generated on the glass microslide by reaction with a 3\% solution of 3-glycidyloxypropyltrimethoxysilane,\textsuperscript{126} which was subsequently used for the immobilization of the amine-modified oligonucleotides in 0.1M KOH.

The epoxylated surface, generated using the above method, was used for amine-modified oligonucleotides and subsequently employed as a universal type of support for the immobilization of modified oligonucleotides.\textsuperscript{126-132} Various nucleophilic and electrophilic group bearing oligonucleotides, viz., mercaptoalkyl, phosphoryl- and thiophosphorylated oligonucleotides were immobilized on epoxy supports with higher immobilization and hybridization efficiency (Figure 13).

In a recent variant of the method, a facile and efficient attachment of oligonucleotides modified with phosphates at their 3'- or 5'-terminal to an epoxylated glass slide has been demonstrated.\textsuperscript{132} The immobilization was achieved under both microwaves and thermal conditions. The constructed microarrays showed high immobilization efficiency (~23\%), signal-to-noise ratio (>98) and high hybridization efficiency (~36\%). The fabricated microarrays also showed high thermal and pH stability and were successfully employed for discrimination of nucleotide mismatches.
Recently, Rozkiewicz et al.\textsuperscript{133} reported an efficient method to immobilize acetylene-modified oligonucleotides on azide-terminated glass substrate by click chemistry via microcontact printing (Figure 14). The immobilization chemistry is irreversible and covalent, resulting in a stable attachment of oligonucleotides, which were subsequently used for hybridization with full-length complementary targets. The strategy involves a single step covalent immobilization that proceeds in high yield without producing any by-product and without the need of a catalyst. Moreover, the resulting triazole link is biocompatible, stable and does not affect bioactivity.

In another approach, Pack et al.\textsuperscript{134} explored the reactivity of oxanine moiety having an O-acylisourea moiety with amine-modified glass substrate for the fabrication of biochips (Figure 15). The strategy involves the introduction of oxa-nucleotide at 5'-terminal of the desired oligonucleotide sequences with or without alkyl-spacers, which were subsequently spotted onto amine-functionalized glass microslides and the spotted slides were incubated at 80°C for 1h in a humid chamber. The resulting microarrays were subjected to hybridization with the labeled target, and the performance was evaluated in terms of immobilization and hybridization efficiency, which were found to be
much superior to that observed in existing methods. The results further displayed higher efficiency of the target recognition by the oligonucleotides bearing a longer spacer (C6 vs. C2 linker). It is inferred from this study that immobilization of oxa-nucleotide bearing oligonucleotides onto the amine-functionalized surface perhaps represents an advanced method for generating DNA conjugated solid surfaces for various applications.

![Figure 14: Immobilization of alkyne-modified oligonucleotides onto azidoalkylated glass surface via ‘click’ chemistry.](image1)

Figure 14: Immobilization of alkyne-modified oligonucleotides onto azidoalkylated glass surface via ‘click’ chemistry.

![Figure 15: Covalent immobilization reaction of oxa-oligonucleotides with amine-functionalized glass surface](image2)

Figure 15: Covalent immobilization reaction of oxa-oligonucleotides with amine-functionalized glass surface.

Yet another elegant approach, describes the tethering of amine-modified oligonucleotides onto tetrafluorophenyl (TFP) activated self-assembled monolayers (SAMs) on gold coated glass slides\textsuperscript{135} (Figure 16). TFP ester was used as an alternative to N-hydroxysuccinimide (NHS) ester because of greater stability of the former under basic coupling conditions. In this methodology, the coupling reactions of amine-modified oligonucleotides with aldehyde, NHS- and
TFP-activated SAMs, and the thiol-modified oligonucleotides with maleimide-terminated SAMs have been evaluated as a function of pH. The thiol-maleimide coupling was found to be pH independent while aldehyde-, NHS- and TFP-activated SAMs exhibited increasing degree of immobilization of oligonucleotide with increasing pH, however, at pH ≥ 10, NHS-activated SAMs showed a decrease in coupling efficiency, which might be due to base-catalyzed hydrolysis of NHS-SAMs that significantly decreased the coupling reaction. Besides, the TFP surface displayed lower background fluorescence and smaller spot radii at each pH. The smaller spot size could be attributed to the hydrophobic nature of the TFP surface. The stability of oligonucleotide arrays was demonstrated by hybridization/dehybridization cycles. There was no significant loss in signal intensity indicating a high degree of stability of the immobilized probes.

Figure 16: Immobilization of modified oligonucleotides onto TFP- (top) and NHS- (bottom) terminated self assembled monolayers (SAMs) on gold coated glass surface

In a protocol involving reversal of sequential steps in the conventional methodology for fabrication of biochips, Razumovitch et al.\textsuperscript{136} have immobilized solution-hybridized amine-modified oligonucleotides onto aldehyde surface via microcontact printing (figure 17). Here, complementary oligonucleotides were first hybridized in solution prior to immobilization onto modified silicon surface. In this manner, the immobilization of the hybridized sequences could prevent
interactions between amino groups on the nucleic bases and the aldehydes on the chip surface. Nevertheless, immobilization occurred through one of the two complementary oligonucleotides bearing 5'-aminoalkyl group. Subsequently, the performance of the constructed array was evaluated by sequential dehybridization and rehybridization of oligonucleotides labeled with fluorescent dyes under laser scanning microscopy. This protocol had advantage of eliminating the hindrances generally encountered during hybridization with the target nucleic acids due to high density of surface attached oligonucleotides.

Figure 17: Immobilization of duplexes on to an aldehyde- modified glass

Limitation of sensitivity has been a major concern for realization of full potential of biochip technology. A general drawback of glass slides is the limited density of the surface silanol groups, used for introduction of modifications due to non-porous nature. This limits the number of reactive groups that can participate in the binding of oligonucleotides, resulting in the poor surface coverage with the probes. A simple way to improve sensitivity of an array is to
increase the surface density to anchor probes or linker molecules. Considering this, Hackler et al. developed six different surfaces with acrylic and epoxy reactive groups for enhanced immobilization efficiency of oligonucleotides. The surface density of the reactive functionalities on glass was found to increase by addition of branched structures. This methodology was subsequently modified by Benters et al. who developed chemically activated glass surfaces for production of microarrays of oligonucleotides, proteins and low molecular weight ligands. The method is based on the attachment of dendritic linker (PAMAM with 64 amino groups in its outer sphere) onto the aminated glass surface, which was subsequently activated with phenylene diisothiocyanate (PDITC) or disuccinimidylglutarate (DSG) to generate a chemically reactive polymeric film covalently affixed to glass slide. The resulting surface was used for the immobilization of amine-modified oligonucleotides. The microarrays prepared were evaluated in terms of hybridization with the labeled targets. The results indicated almost 2-folds greater surface coverage with the captured oligonucleotides than that with the conventional microarrays bearing linear linkers as the dendrimers coating permits the immobilization of many more probes because of numerous functionalized groups’ presence in the structure. Further, the surfaces were found to be resistant to repeated alkaline regeneration procedure, which is likely due to cross-linked polymeric structure of the dendrimers film. The higher stability of the surface allowed multiple hybridization experiments without significant loss of signal intensity. Also, a dendrimer coat acts as a spacer between probe and the substrate surface, which facilitates efficient binding and accessibility of the immobilized probe to target. In a diverse approach, Lim et al. developed an improved protocol for the fabrication of DNA biochip with PAMAM dendrimers peripherally modified with biotin and avidine. Biotinyl-oligonucleotides were tethered to the surface showing significantly improved sensitivity, specificity and stability of the biochip. A 4-fold increase in fluorescence intensity of the spots, excellent specificity for discriminating single nucleotide polymorphism with detection limit in the range of 1pM to 1nM were some of the distinctive features of the protocol.
Recently, an exquisite nanoscale-controlled surface has been reported by Hong et al.\textsuperscript{140} This strategy not only ensured proper spacing between immobilized biomolecules but also revealed good discrimination efficiency in a wide temperature range (37 - 50°C). In this strategy, a cone-shaped dendron ensures adequate spacing between immobilized oligonucleotides on surface, which ultimately provides sufficient accessibility for the incoming target DNA, almost similar to solution-like conditions (Figure 18). Hybridization efficiency was scored in the range of 80-100% (vs. \textasciitilde20-35% usually obtained in other reported protocols.

As described above, several surface chemistries have been shown to immobilize pre-synthesized oligonucleotides in an orientation-specific manner. These methodologies must be compatible with the nanolitre scale volumes of oligomer reagents which contact the array over a small portion of their surface. In this regard, Lee et al.\textsuperscript{141} have proposed that a three dimensional surface would provide greater surface contact and higher binding efficiency. The group has developed a cross-linked polyethylenimine coated glass surface that offered exceptional binding and hybridization characteristics.

In another protocol, to provide high capacity surfaces, chitosan, an amine-rich polysaccharide, coated glass slides were used for tethering biomolecules including synthetic oligonucleotides. The surface amino groups were first activated with phenylene diisothiocyanate (PDITC) to generate reactive isothiocyanato functions on the surface which were subsequently reacted with amine-modified oligonucleotides. The resulting surface exhibited high loading capacity for oligonucleotide immobilization and good binding.
efficiency, and enabled excellent availability of the surface-bound probes for hybridization with the targets and effective detection of single nucleotide polymorphisms (SNPs) with low background. Surface attached oligonucleotide probes revealed highly specific hybridization properties.

Recently, Wu et al.\textsuperscript{142} compared the immobilization of unmodified oligonucleotides (26-70mer) and 5'-amine-modified oligonucleotides onto aminoalkylated glass slides modified with acrylic acid-co-acrylamide copolymer under the influence of UV light and in the presence of EDC/NHS, respectively (Figure 19). The investigators observed no significant difference in the hybridization efficiency of the immobilized amine-modified and unmodified oligonucleotides on the dendritic surface. The results indicate that unmodified oligonucleotides, being cost-effective, can be used for immobilization on the modified glass surface.

Figure 19: Immobilization of 5'-amine-modified oligonucleotides onto poly (acrylic acid-co-acrylamide) copolymer film on glass microslide
More recently, in an elegant approach, modified analogs, hexitol (HNA) and altritol nucleic acids (ANA) with high affinity towards DNA and RNA targets, were employed for the preparation of biochips.\textsuperscript{143} Immobilization of these chimeric nucleic acids was effected by reaction of diene-modified oligonucleotides with maleimidoalkyl-activated glass slides (Figure 20). The distinguishing features of the strategy are the higher match/mismatch discrimination of ANA and HNA arrays, which might be due to improved affinity of the probes and higher stability of the resulting duplexes (particularly with respect to RNA). The relative intensity of the signal and match/mismatch discrimination increased up to 5-folds for DNA targets and up to 3–3.5-fold for RNA targets applying HNA or ANA arrays. The features accrue from a pre-organization (entropy factor) and enzymatic stability. The constructed biochips can be stored for a longer duration and reused due to higher chemical and enzymatic stability of HNA and ANA oligomers.

\vspace{1cm}

![Figure 20: Immobilization of ANA and HNA onto maleimidoalkyl-glass surface](image)

Figure 20: Immobilization of ANA and HNA onto maleimidoalkyl-glass surface
In another approach, Dendane et al. reported an efficient surface patterning of oligonucleotides onto the inner wall of fused silica capillary tubes as well as on the surface of glass microslides through oxime bond formation. Their strategy involves the preparation of 2-(2-nitrophenyl) propyloxycarbonyl (NPPOC) protected aminooxy silane, which was used for functionalization of glass surfaces. On exposure to UV light, NPPOC group was removed and aldehyde-modified oligonucleotides reacted with the unmasked aminooxyalkylated glass surface through stable oxime bond formation obviating the need for chemical reduction with sodium cyanoborohydride. The projected approach offers an alternative methodology for the immobilization of bioactive molecules in the microchannels of “labs on chip” devices.

An overview of the attachment chemistries employing covalent linkage (photochemical and thermochemical) for patterning of arrays is given in Table 8.

Table 8: Some commonly used chemistries for immobilization of pre-fabricated oligonucleotides on glass surface via covalent method

<table>
<thead>
<tr>
<th>S.No</th>
<th>Functional Group On Glass</th>
<th>Functional Group On Oligomer</th>
<th>Chemical Method</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Maleimide</td>
<td>Cinnamide</td>
<td>Photochemical</td>
<td>[25]</td>
</tr>
<tr>
<td>2.</td>
<td>-(CH₂)ₙ</td>
<td>Anthraquinone</td>
<td>Photochemical</td>
<td>[108]</td>
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## Chapter I

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<td>UV (2+2 photoaddition)</td>
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1.7 **Scope of the Present Work**

From the above literature survey, it is evident that in the post-genomic era, synthetic oligonucleotides are finding a wide variety of applications in the area of genomics, molecular biology and medicinal chemistry. This has led to an increase in demand of chemically synthesized synthetic oligonucleotides (both modified and unmodified). The field of nucleic acid chemistry has been revolutionized due to this exponential increase of synthetic nucleic acids. Newer methods and techniques have been developed to synthesize, purify and store these biomolecules more efficiently, in a cost-effective manner.

Fabrication of microarrays is among major applications of modified synthetic oligonucleotides. Currently, oligonucleotide-based microarrays are frequently used in a variety of biomedical applications such as mutation detection, disease diagnosis, gene expression analysis etc. The technology includes synthesis and immobilization of modified oligonucleotides onto solid substrates. The immobilized oligonucleotides are subsequently hybridized with labeled target, which could be exploited for diagnostic purposes. The quality of constructed microarrays depends upon number of parameters, viz., choice of support, immobilization chemistry, surface density of the immobilized probes, concentration of the target, detection sensitivity, mismatch discrimination and signal to noise ratio, etc. With the introduction of laser dyes and quantum dots for the labeling purposes, better quality glass surfaces and improved attachment chemistries, the microarray technology has improved significantly. Further improvements in the present methodologies for oligonucleotide synthesis, attachment of probe onto substrate and detection of the target molecule, would be advantageous in making these arrays more accessible.

The present study was undertaken with an outlook to develop facile, rapid and inexpensive protocols for the construction of oligonucleotide-based microarrays that can be employed for disease diagnosis and molecular biology.
1.8 References


