

Current Challenges in Nucleic Acid Synthesis

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Abstract: Our understanding of the role of nucleic acids in regulating the process of life is growing rapidly. As researchers delve deeper into the cellular activity of molecules such as microRNA, small interfering RNA, and long non-coding RNA, the oligonucleotide chemist is confronted with the challenge of synthesizing the tools needed to continue this work. Furthermore, new applications are being developed

that require increasingly complicated oligonucleotide constructs. In this review, we explore the current challenges confronting oligonucleotide synthesis, describe the chemistry and the enzymology being developed in response to those challenges, and discuss future challenges as-yet unmet.

Keywords: DNA · nucleic acids · oligonucleotides · RNA · synthetic biology

1. Introduction

Synthetic oligonucleotides are now considered by many scientists to be a commonplace molecule: a commodity readily ordered from many sources, from which a reliable product can be expected in a timely fashion. This is largely true because the vast majority of oligonucleotides ordered are DNA primers and probes for polymerase chain reactions (PCRs); a tool utilized by a large number of scientists in a variety of fields. The need for cheaper oligonucleotides in increasing quantities led to the development of the oligonucleotide “mega houses” that produce huge numbers of inexpensive oligonucleotides on a daily basis. In general, the quality of these inexpensive oligonucleotides is adequate for most scientific applications.

In the 1990s, there was a general shift in scientific development from the hands of chemists into the hands of engineers, whose main directive was to continue to develop inexpensive, high-throughput methods.^[1] Improvements in methodology were largely directed towards multi-parallel automation and decreasing the cost of synthesis, largely by shortening the overall cycle time and decreasing the amount of reagent used. This was mostly accomplished through the reduction of the scale of the synthesis from the classic 1 and 0.2 μmol scales to scales as low as 5 nmol. This shift to less expensive oligonucleotides and the accompanying overall decrease in attention to chemical integrity was largely justified by the needs of the day.

During that time, innovations in the chemistry of making oligonucleotides were mostly directed towards the anticipated large-scale production of therapeutic antisense oligonucleotides, many of which contained phosphorothioate internucleotide linkages and 2' O-methyl-modified nucleoside units.^[2] The need for significantly lower costs continued to require the development of

novel methodologies, while investigations into the use of older methods, such as solution-phase chemistry, sought to unearth previously used methodologies that may not have been acceptable for high-throughput use, but might be practical for use at a large scale. With a few exceptions, such as work by Beaucage et al. to develop a thermolytic approach to making oligonucleotides aimed for the synthesis of microarrays,^[3] the majority of research in oligonucleotide chemistry in that era focused on large-scale process development.

However, at the turn of the millennia, a surge in the development of powerful new tools to study molecular and cellular biology, combined with the need for more highly modified oligonucleotides by the therapeutic field, justified continued research in the development of modified oligonucleotide chemistry. While the diagnostic industry began exploring various solutions to single-nucleotide polymorphism (SNP) detection that required more complicated oligonucleotides, the continued growth in aptamer research expanded the need for longer oligonucleotides, both RNA and DNA, unmodified and modified. This need for more sophisticated oligonucleotides was likewise driven by those exploring novel approaches to next-generation sequencing, such as the Life Technologies' SOLiDTM (Sequencing by Oligonucleotide Ligation and Detection) platform. Additionally, the introduction of siRNA and miRNA reinvigorated research into the

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synthesis of RNA, with particular interest in the introduction of modified nucleotides. In every application, the importance of obtaining high-quality oligonucleotides became increasingly apparent as the boundaries of sensitivity, accuracy, and chemical complexity were continually redefined. Additionally, as the biology of RNA continues to unravel, the preparation of modified RNA to further those studies has become more important.

In many ways, the pendulum has swung back towards the need for innovative chemistry to synthesize increas-

ingly complicated oligonucleotides for use in increasingly complex applications. After a short introduction to the chemical synthesis of oligonucleotides from a historical perspective, we explore recent advances in oligonucleotide synthesis in the context of newly developed biological applications.

2. A Short History of Oligonucleotide Synthesis

There are many excellent reviews about the historical development of oligonucleotide chemistry, as well as specific aspects of the chemistry, a number of which we list in the references.^[4] Below is a very brief summary of the most critical steps that led to the most commonly used methods to make oligonucleotides today.

2.1. The Path to Phosphoramidite Oligonucleotide Chemistry

In 1955, Michelson and Todd published the first account of the chemical synthesis of an oligonucleotide; a dithymidyl nucleotide.^[5] The phosphate link between two thymidines was made by first preparing the 3'-benzylphosphoryl chloride of a 5'-protected thymidine using benzylphosphoryl dichloride. Subsequently, this was then reacted with the 5'-hydroxyl of a 3'-protected thymidine to make a dinucleotide (Scheme 1).

Then, in the 1950s, Khorana et al. made two significant contributions that made possible the synthesis of oligonucleotides long enough to be biologically relevant. His first contribution was a practical scheme for the synthesis of oligonucleotides, called the phosphodiester method of oligonucleotide synthesis^[6] (Scheme 2). This method was revolutionary at the time and produced a truly remarkable feat: the synthesis of a DNA template that was used to transcribe a 72mer tRNA molecule.^[7]

Khorana et al. also developed the oligonucleotide protection/deprotection scheme^[8] that is still used today more or less intact. The 4,4'-dimethoxytrityl (DMT) protecting group for the protection of the 5'-hydroxyl^[8b] group is ubiquitous in oligonucleotide chemistry. Khorana et al. introduced protecting groups for the nucleosidyl exocyclic amines that are known today as standard protecting groups: isobutyryl (iBu) for guanosine, and benzoyl (Bz) for adenosine and cytidine.^[8a,9] Although others exist,^[10] these groups are those most commonly used, with the possible exception of acetyl-protected cytidine, which is more readily and cleanly removed compared with Bz^[11] (Figure 1).

The next significant steps forward were taken by Letsinger, of Northwestern University, who began his work on biomacromolecules with peptide synthesis. Letsinger and Kornet developed a peptide synthesis method using solid-phase chemistry that utilized flow-through technology with a cyclic chemistry scheme of adding units sequen-

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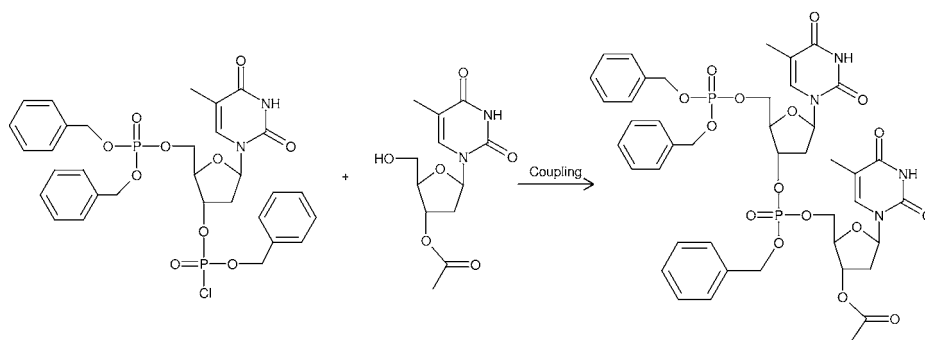


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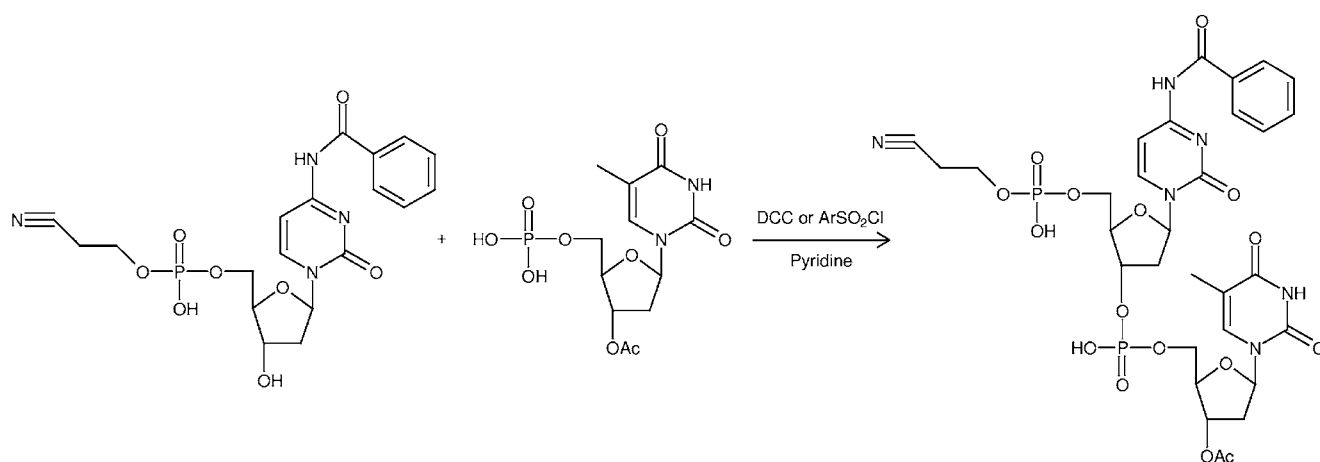


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Scheme 1. First chemical synthesis of thymidine dinucleotide.



Scheme 2. Phosphodiester approach to the synthesis of oligodeoxynucleotides developed by Khorana and co-workers. DCC = *N,N'*-dicyclohexylcarbodiimide, ArSO_2Cl = aryl sulfonyl chloride.

tially.^[12] They later applied this method to the cyclic oligonucleotide synthesis procedure taught by Khorana.

In 1965, Letsinger and Mahadevan published the first paper^[13] describing the solid-phase synthesis of dimer and trimer oligonucleotides using the same “popcorn” polymer support described for use in peptide synthesis.^[12,14] The support consisted of a styrene–divinylbenzene polymer that had the unfortunate property of swelling in particular solvents. Nonetheless, the ability to filter off reagents instead of having to isolate the product after each chemical reaction was a critical step forward in the development of the chemistry.

Then, in the late 1960s, 12 years after Michelson and Todd's publication of the synthesis of dithymidine through a phosphate triester method,^[15] Letsinger reintroduced a phosphotriester method of oligonucleotide synthesis based on the chemical activation of terminal diester phosphates of nucleotide derivatives^[16] (Scheme 3). Although several phosphorus protecting groups were explored, the β -cyanoethyl group was the most commonly used, primarily because it was easily removed with ammonium hydroxide.^[17] The phosphate was activated with

a reagent such as mesitylenesulfonyl chloride (MSCl) or mesitylenesulfonyl nitrotriazole (MSNT), which were the coupling reagents of choice.^[17,18]

Combined with solid-phase chemistry, the phosphotriester method was the first chemistry amenable to automation and several companies, such as Vega Biotechnologies and Biosearch Technologies, introduced DNA synthesizers based on this method. These innovations offered the biologist a new palate of readily accessible tools, synthetic oligonucleotides, that directly led to the development of the next generation of molecular biology techniques, most notably PCR.

However, the phosphotriester method still suffered from critical drawbacks. Among them were the relatively low coupling efficiencies, which made oligonucleotides longer than 20 bases in length difficult to synthesize, and the long cycle times. The coupling time was eventually decreased by the use of improved catalysts,^[19] but by this time the phosphotriester chemistry was already displaced by the next major advancement in oligonucleotide chemistry.

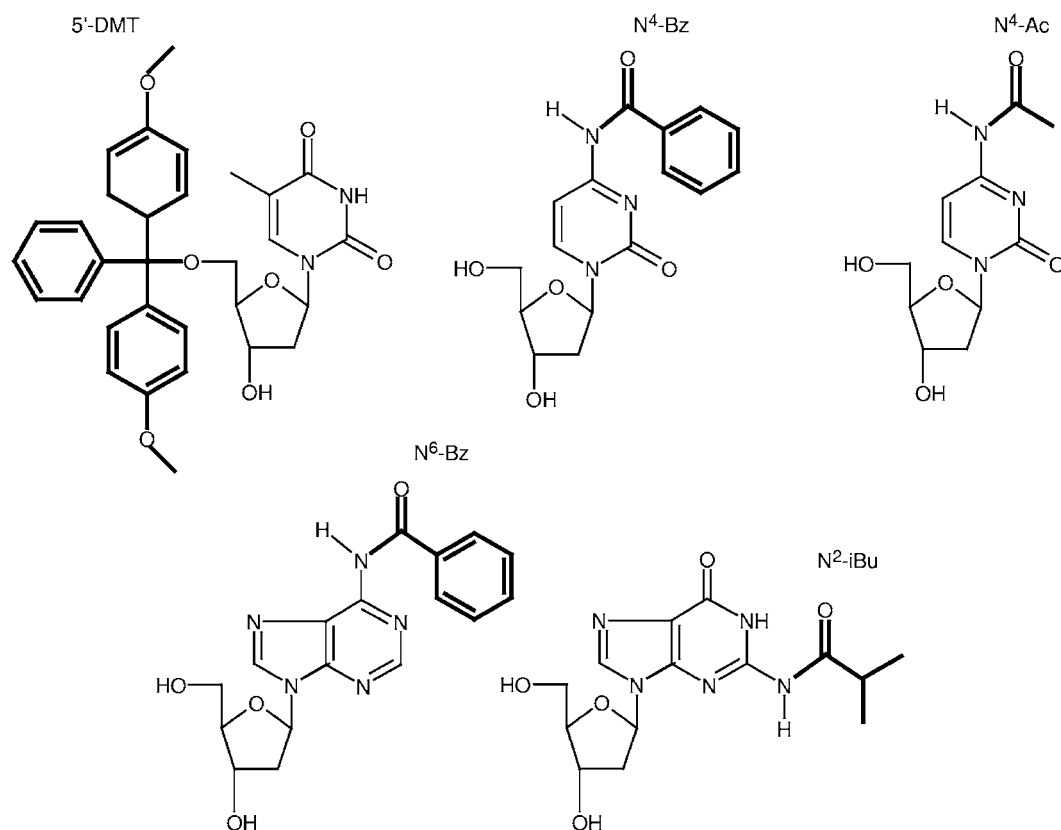
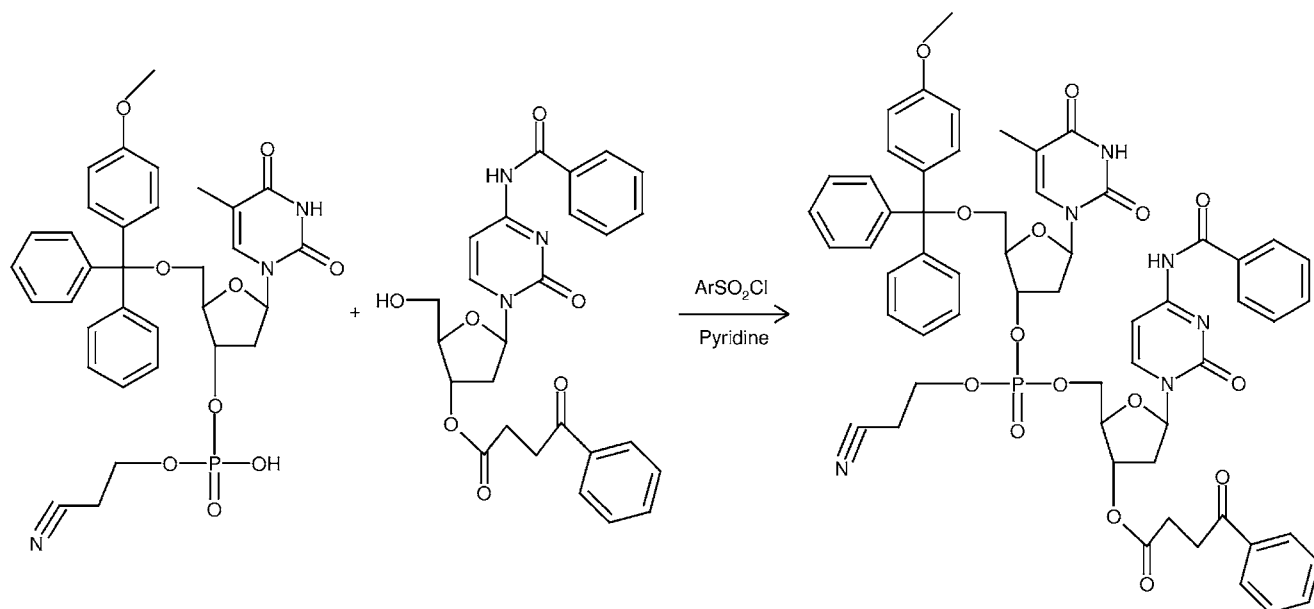


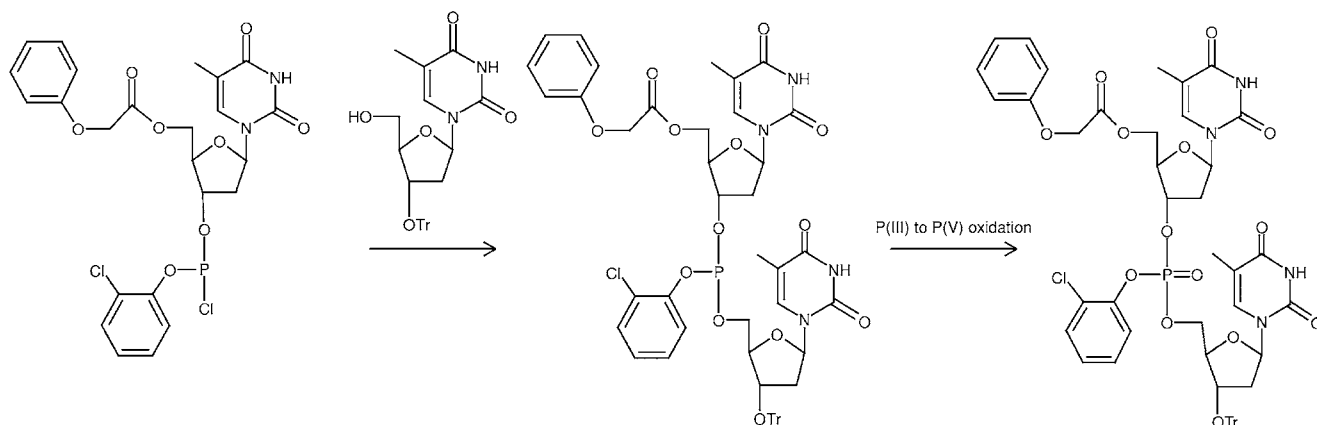
Figure 1. Most common sugar and base protecting groups suggested by Khorana et al. for oligonucleotide synthesis. Structures of protecting groups are shown in bold.



Scheme 3. Phosphate triester method of oligonucleotide synthesis developed by Letsinger and co-workers.

In the mid-1970s, Letsinger described the phosphite triester method of oligonucleotide chemistry^[20] (Scheme 4). This chemistry uses a coupling reagent where

the phosphorus is in the P(III) state, instead of the classic P(V) phosphorus state used in earlier methods. The new scheme required an oxidation step in the synthesis cycle



Scheme 4. Phosphite triester approach to the synthesis of oligodeoxynucleotides developed by Letsinger and co-workers. Tr=trityl.

to prepare the natural P(V) backbone. The major advantage of this chemistry was a significant reduction in the time required for coupling, primarily due to the highly reactive nature of the nucleoside phosphomonochloridite intermediate.

The early form of phosphite triester chemistry did have major drawbacks, most notably the susceptibility of the nucleoside phosphomonochloridite intermediate to hydrolysis. A method was eventually developed that led to the production of a DNA synthesizer with a cycle time of 15 minutes or less using the phosphodichloridite method.^[21] Unfortunately, this discovery was too late to save this particular form of phosphite triester chemistry.

Caruthers, a protégé of both Letsinger and Khorana, made the next significant advancements to the science of oligonucleotide synthesis. Caruthers was well trained for his place in history and he told his own story in a recent article.^[22] In 1981, he and Matteucci published the use of inorganic silica gel matrices as supports for oligonucleotide synthesis,^[23] which resolved the problems with swelling of the support. More recently, there has been a return to the polystyrene polymers of old; however, they are greatly improved.^[24] These polymers allow much greater loading and provide improved washing properties in many instrument formats; therefore, having a profound impact on the cost of mid to large-scale synthesis of oligonucleotides.

Caruthers' other significant contribution was the simple replacement of the chloride leaving group on the phosphite with a dialkylamino group.^[25] This resulted in a stable phosphitylating reagent called a nucleoside phosphoramidite that could be made in advance and stored until needed. The nucleoside phosphoramidite was activated just prior to a coupling reaction by simply adding a weak acid, such as tetrazole. The resulting oligonucleotide intermediate is a phosphite triester, so oxidation is still required. This subtle, yet critical, change was paramount for the advancement of the industry. Instruments

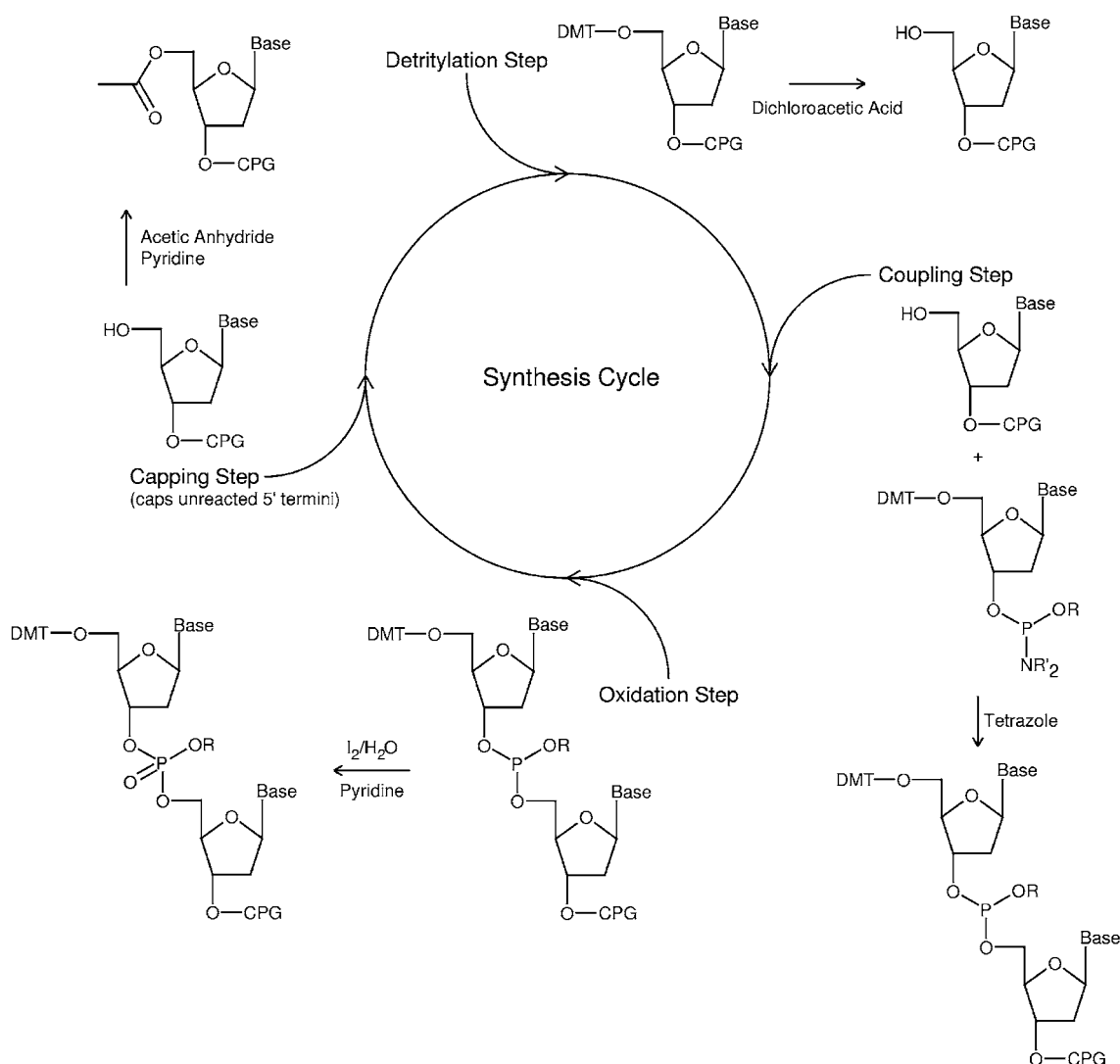
were rapidly developed that took advantage of this new chemistry.

Besides the steps of 1) deblocking of the 5'-hydroxyl group, followed by 2) activation and coupling of the nucleoside phosphoramidite to the 3'-support-bound growing oligonucleotide, and 3) oxidation, an additional step to 4) cap any chains that failed to elongate was added. This method, the "synthesis cycle" as it is generally referred to, has remained more or less unchanged for the last three decades (Scheme 5).

2.2. H-Phosphonate Approach

Though the first synthesis of an oligonucleotide dimer using H-phosphonate chemistry was described by Hall, Todd, and Webb in 1957,^[15] it was not explored further until the mid-1980s when Froehler and Matteucci,^[26] and Stawinski et al.^[27] developed oligonucleotide synthesis through the H-phosphonate method on a solid support (Scheme 6). Since that time, the H-phosphonate method has been optimized and adjusted for the synthesis of oligodeoxyribonucleotides and, especially, oligoribonucleotides allowing for the reliable synthesis of long oligomers (up to a 50–60mer).^[28]

The H-phosphonate approach has two significant advantages over the phosphite triester method: it does not require oxidation after each coupling step, nor does it require a phosphorus protecting group. Thus, the oxidation of all H-phosphonate internucleotide linkages can be performed at the end of the synthesis of the oligonucleotide. Moreover, the internucleotide H-phosphonate linkages can be oxidized into normal phosphodiester,^[26a,29] phosphorothioate,^[26a,30] phosphoroamidate,^[26a,30–32] or phosphotriester^[26a] backbones. Unfortunately, the method was difficult to translate across many platforms, requiring extensive development to be used successfully. Therefore, despite significant improvements in H-phosphonate chemis-



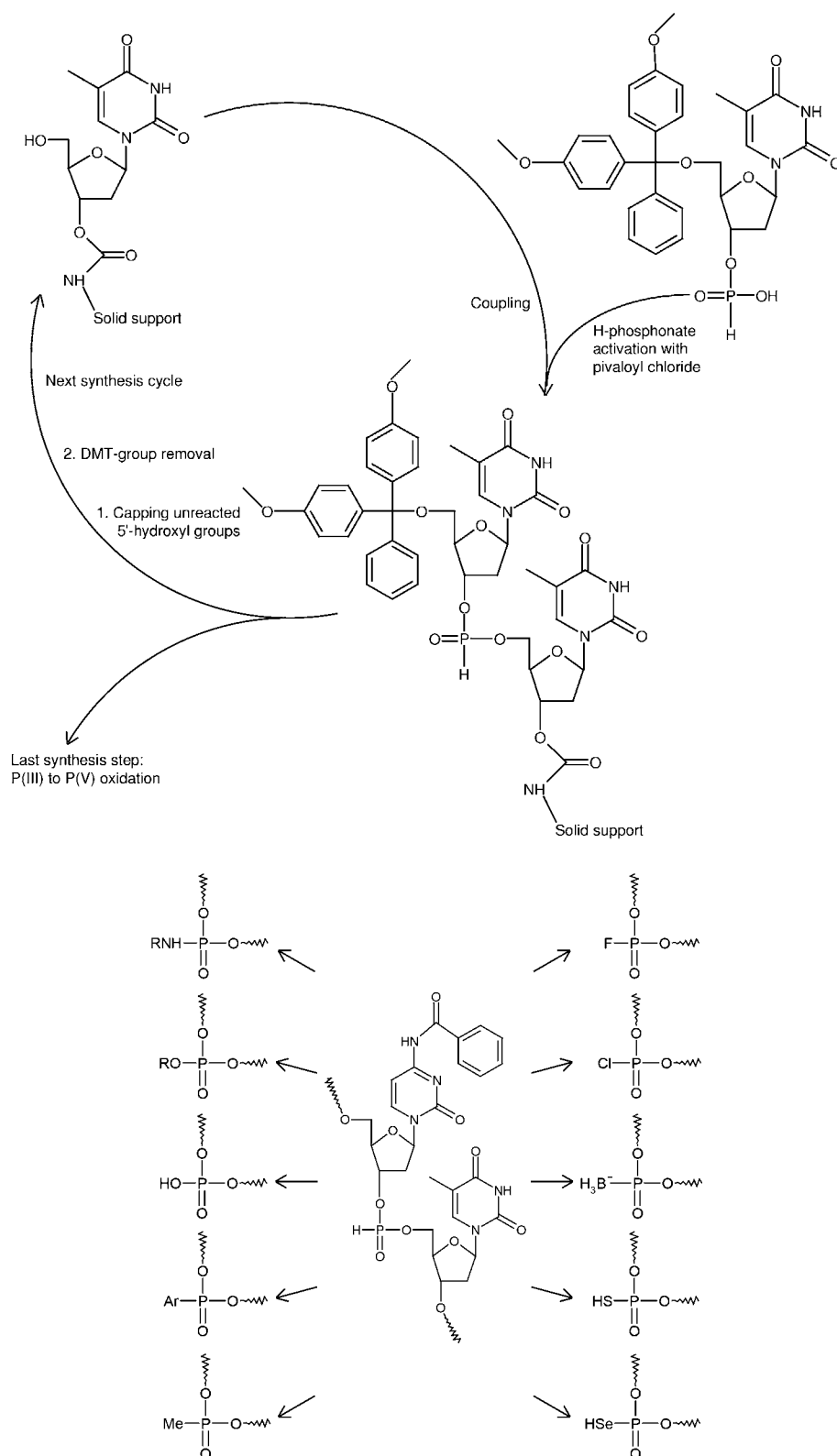
Scheme 5. The synthesis cycle using the phosphoramidite method for synthesis of oligonucleotides developed by Caruthers and co-workers. Reprinted with permission from Trilink Biotechnologies. http://www.trilinkbiotech.com/tech/oligo_history.pdf

try and novel coupling reagents,^[33] this approach is not as commonly used as the phosphoramidite approach.

2.3. Enzymatic Synthesis

The enzymatic synthesis of short oligoribonucleotides was developed in parallel with the chemical synthesis of oligodeoxyribonucleotides. The first major step towards the development of enzymatic preparation of oligoribonucleotides was made by Heppel et al.,^[34] when they noticed that under specific conditions a pancreatic RNase could perform a reverse reaction and generate an internucleotide 3'–5' linkage between a nucleoside-2',3'-cyclic phosphate and an unprotected nucleoside. This route was optimized to produce preparative amounts of short oligoribonucleotides for larger scale molecular biology studies.^[35]

However, the real breakthrough in the enzymatic synthesis of oligo- and, especially, polynucleotides was made after the groups of Grunberg-Manago and Ochoa isolated and characterized polynucleotide phosphorylase (PNP).^[36] In the forward direction, the enzyme catalyzes the formation of oligo- and polyribonucleotides from nucleoside 5'-diphosphates.^[36a,37] In the reverse reaction, polyribonucleotides are phosphorylated to yield nucleoside 5'-diphosphates (ppN).^[38] Later, it was found that PNP could use 2'-deoxyribonucleoside-5'-diphosphates (ppdN) as a substrate; thus making it possible to synthesize oligodeoxyribo- and polydeoxyribonucleotides.^[39] Subsequently, Singer et al. made the important discovery that short oligonucleotides could serve as primers for PNP.^[40] Nirenberg and Matthaei used these polyribonucleotides for their work on the genetic code.^[41]



Scheme 6. H-Phosphonate approach to the synthesis of oligonucleotides: main synthetic cyclic scheme (top) and conversion of H-phosphonate intermediates to oligonucleotide derivatives with various modifications of internucleotide linkage (bottom).

Due to the processive nature of PNP,^[42] it was difficult to incorporate just a single nucleoside 5'-diphosphate (ppN or ppdN). Only under carefully controlled conditions was it possible to successfully elongate oligonucleotides with discreet sequences up to 13 bases in length.^[43] A solution to this polymerization problem was reported, where the 3'-hydroxyl of the ppN was protected; thus blocking further elongation of the oligonucleotide after a single incorporation.^[44] The 3'-protecting group can be removed by a mild chemical or enzymatic treatment or by UV light under neutral conditions and the synthesis of the oligonucleotide can be continued through subsequent addition of desired nucleotides. However, this approach was never optimized and deemed to be inefficient for the synthesis of longer oligonucleotides.

In the 1970s, RNA ligase was used to join strands of oligoribonucleotides and oligodeoxyribonucleotides to synthesize longer RNA and DNA molecules with moderate yields.^[45] Protection of the 3'-OH group of the donor strand was necessary to avoid donor-strand polymerization and/or cyclization in the case of longer oligonucleotides. Acceptor oligonucleotides with modified bases at the 3'-end were used as substrates for RNA ligase in the preparation of longer oligonucleotides with internal modifications;^[46] thus demonstrating a powerful combination of chemical and enzymatic approaches for the preparation of DNA and RNA sequences.

In the late 1980s, Mandecki and Bolling described an approach to gene synthesis that introduced about 100 base pair (bp) inserts in between two FokI restriction digestion sites in a plasmid.^[47] This was followed by several self-priming PCR approaches^[48] (Figure 2) and by assembly PCR in the early to mid 1990s.^[49] Assembly PCR, which uses DNA polymerase and a series of short oligonucleotides (≈ 40 nucleotides (nt)), allowed for the preparation of a 1.1-kb fragment from a total of 56 oligonucleotides. The assembly PCR technique further matured over the years and was improved to the point where it

can now be accomplished with fewer thermal cycles^[50] and is accompanied by software algorithms to assist in oligonucleotide design.^[51] At present, the development of technologies for gene synthesis is rapidly evolving and includes sequence assembly from microchips,^[52] more stringent consideration of the assembly process,^[53] and the development of error-correction technologies for gene synthesis.^[54] The fast development of reliable gene synthesis techniques,^[55] coupled with the flurry of research in the area of genomics, has led to the establishment of commercial entities that allow researchers to simply order their gene(s) of choice.^[56]

3. Application Driven Chemistry

The more recent innovations in oligonucleotide chemistry have been largely applications driven. The applications themselves have become more demanding, requiring more highly modified oligonucleotides. For the most part, the phosphoramidite method has held sway, however, the types of molecules that have to be prepared on a routine basis has multiplied tremendously. A more elegant chemistry solution was often needed and some of the innovations introduced to reduce turnaround times and costs had to be reversed to successfully prepare certain constructs. A number of the applications that have helped drive the current directions of oligonucleotide chemistry are described below, along with some of the hurdles confronted by the oligonucleotide chemist and the innovative chemistries and enzymology designed to overcome those problems.

3.1. In Situ Microarray Synthesis

An area where a great deal of chemical innovation took place during the 1990s was in the science of synthesizing oligonucleotides directly on DNA microarray chips.^[57] The challenge was to perform the oligonucleotide synthesis on a solid support with extremely high efficiency and precision, since on-support synthesized oligonucleotides cannot be post-synthetically purified.

The first approach to in situ synthesis of a microarray was based on light-directed oligonucleotide synthesis with photolithographic masking.^[58] In this method, the surface of the solid support is modified with a linker containing a photolabile hydroxyl-protecting group. Laser light induces OH deprotection at very small but defined sections of the chip while the rest of the chip is masked. The coupling step is performed with phosphoramidite monomers containing a photolabile 5'-protecting group. After P(III) to P(V) oxidation and replacement of the photolithographic mask, the whole cycle is repeated many times to allow the fabrication of discreet clusters of oligonucleotide sequences at defined positions on the chip (Figure 3).

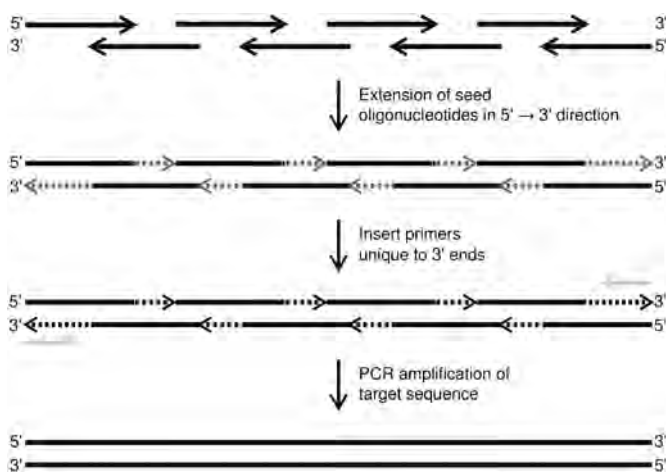


Figure 2. A general representation of steps used in assembly PCR.

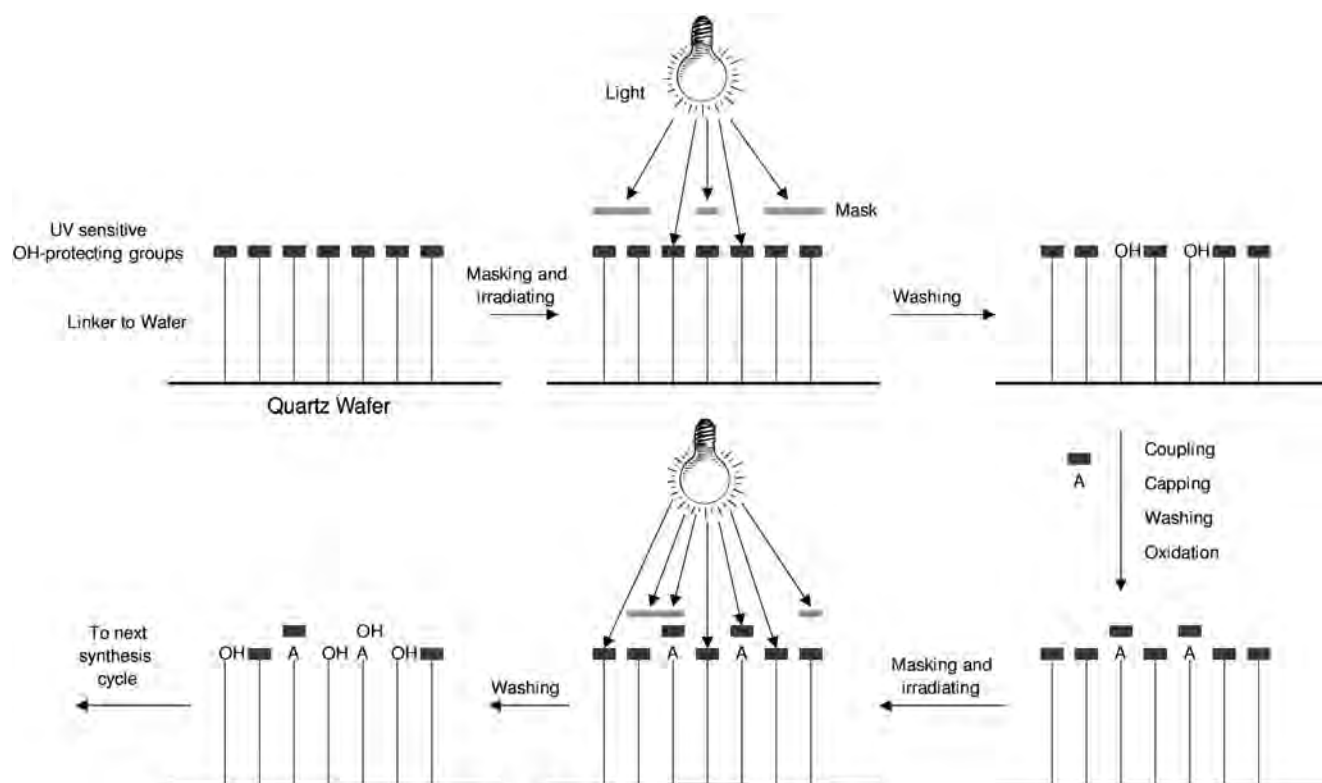


Figure 3. Affymetrix light-directed, on-chip oligonucleotide synthesis with photolithographic masking.

Affymetrix Inc., was one of the pioneers who developed a microarray fabrication process with phosphoramidite monomers containing a photolabile 5'-hydroxyl protecting group. A number of different photolabile protecting groups were also explored by Affymetrix and others.^[59] Even though neither the photochemistry nor the masking method was completely optimal, Affymetrix's mask lithography technology produced high density and high-quality chips with an extremely high number of probes (millions of oligonucleotide probes per chip).

To overcome the technological issues with the preparation and use of fixed masks and to reduce the cost of microarray fabrication, a new maskless technique for the light-directed synthesis of oligonucleotide microarrays was introduced^[60] and used a digital micromirror array to form virtual masks. These mirrors are individually addressable and can be used to create any given pattern or image over a broad range of wavelengths, theoretically allowing the synthesis of millions of different oligonucleotide sequences in an 1 cm² area. Further optimization of this technology yielded a process that produced very high quality oligonucleotides on chips.^[61]

Both of these technologies rely on expensive phosphoramidite monomers with photolabile groups for the protection of the 5'-hydroxyl function. To accommodate the use of common 5'-DMT-phosphoramidite monomers in light-directed microarray synthesis, it was suggested to use photogenerated acids to remove the 5'-DMT group.^[62]

This approach suffered from the poorly controlled kinetics of DMT deprotection and led to low efficiency of oligonucleotide synthesis.

Another method for the direct synthesis of oligonucleotides on chips was developed by Agilent Technologies and others.^[63] The *in situ* synthesis is performed by an inkjet oligonucleotide synthesizer that prints the desired phosphoramidite nucleosides onto the appropriate location of the chip, with common processing of each added nucleotide (Figure 4). This technology uses standard phosphoramidite chemistry, relies on the effectiveness and precision of delivery of the reagents, and allows the preparation of fairly long oligonucleotides (≈ 60 –70mer) in high quality on a support with a density of about 10^5 – 10^6 probes per chip. Further technological improvements suggest that this approach can be used to cleanly prepare oligonucleotides up to 150 bases in length directly on a chip.^[64]

Furthermore, *in situ* technology was developed that utilized a semiconductor-based oligonucleotide synthesis technology to manufacture custom microarrays.^[65] The oligonucleotides are synthesized on an array of prefabricated microelectrodes on a semiconductor chip using phosphoramidite chemistry under electrochemical control. The electrochemical reaction is activated at specific electrodes on the chip, which then generates protons. This, in turn, removes the blocking group from the 5'-hydroxyl group; thus allowing oligonucleotide synthesis to

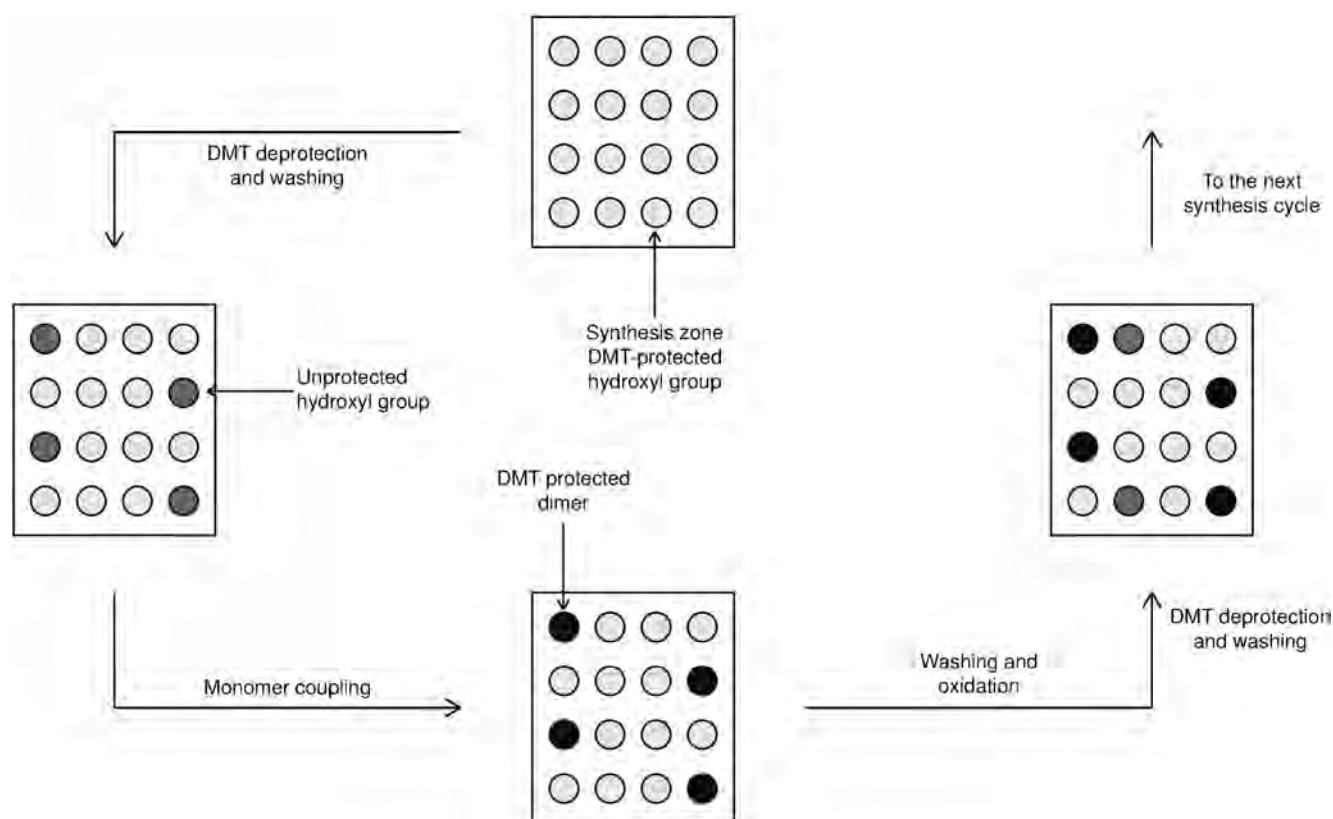


Figure 4. Agilent on-chip synthesis of oligonucleotides using an inkjet synthesizer.

take place at a specific location of the microelectrode. Up to 10^5 – 10^6 probes per chip can be prepared by this technology. Since these microarrays use addressable microelectrodes, it is possible to detect DNA hybridization on these chips electrochemically.^[66]

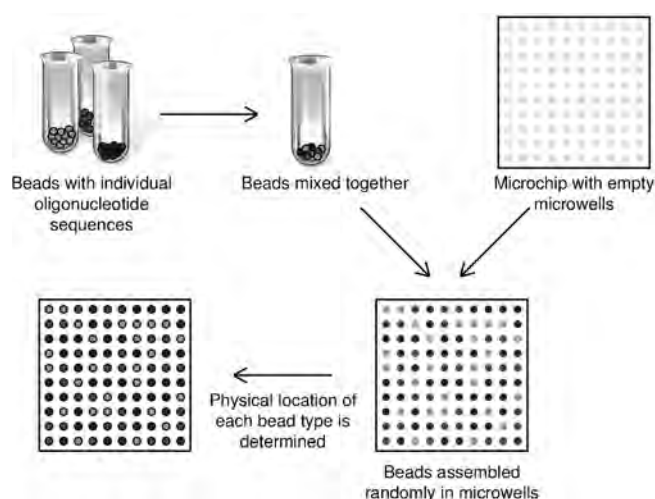


Figure 5. Illumina microarray platform using BeadArray™ technology.

Illumina Inc., has developed a novel microarray platform using BeadArray™ technology that uses conventional phosphoramidite chemistry to synthesize oligonucleotides on 3 μm silica beads^[67] (Figure 5). After deprotection, the oligonucleotide remains attached to the bead. The mixture of beads, containing thousands of oligonucleotide chains with specific sequences on each bead, is assembled randomly into wells. Statistically speaking, each bead with its unique oligonucleotide sequence is represented with an average 30-fold redundancy on each microchip array. Although each chip has its own unique distribution of oligonucleotide sequences, the spatial position of each sequence is decoded by the manufacturer and provided with every chip. Microarrays are still an important part of today's molecular biology toolkits, although they appear to have reached a plateau and seem to be losing market share to novel sequencing technologies.

3.2. RNA Synthesis

Although the synthesis of RNA oligonucleotides has a history as long as the synthesis of DNA oligonucleotides,^[8b,68] difficulties in making these molecules made the commercialization of RNA synthesis follow that of DNA by about a decade. The problem was primarily with the

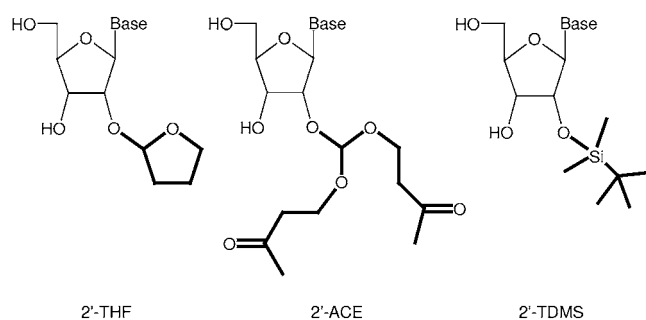


Figure 6. Structures of some common 2'-protecting groups for oligoribonucleotide synthesis. Structures of protecting groups are shown in bold.

protection of the 2'-hydroxyl group of the ribonucleoside. RNA is very base labile and undergoes general base-catalyzed hydrolysis under fairly mild basic conditions.^[69] The normal deprotection reagents used for DNA rapidly degrade RNA oligomers with free 2'-OH groups within minutes. The solution generally adopted was to protect the 2'-hydroxyl with a group that could withstand the base treatment required to remove the exocyclic amino and phosphorus protecting groups. The 2'-hydroxyl group is deprotected last under non-basic conditions. At that point, RNA becomes susceptible to base hydrolysis and degradation by RNases; enzymes considered more or less ubiquitous in our environment.

There were two main directions of thought regarding how to protect the 2'-hydroxyl groups. Ogilvie, a former graduate student of Letsinger, introduced silyl protection, whereby the silyl group is removed with fluoride ions under neutral conditions (Figure 6). The other approach was developed mainly by Reese, who used acid-labile protecting groups, exploring acetals for the most part. An excellent review, written by Beaucage, documents their efforts in detail.^[70] There were other approaches as well, such as the 2'-ACE method, which used a 2'-*O*-bis(2-acetoxyethoxy)methyl (ACE) protecting group removed by the end user by simple treatment with mild acid.^[71] An early, and perhaps first commercial source of RNA monomers in the US, Glen-Midland Research, used a tetrahydrofuran protecting group that required acidic deprotection.^[8b,72] However, the most limiting factor was the lack of general interest. Few researchers were working with synthetic RNA at the time.

3.2.1. Early Applications—RNA as a Target

Walder, of the University of Iowa, was one of the researchers studying RNA during the late 1980s, investigating the biological role of the endonuclease, ribonuclease H (RNase H). He used synthetic RNA as targets for antisense DNA molecules targeted against mRNA. He and others discovered that RNase H and its various isozymes were responsible for the antisense results many research-

ers were observing.^[73] Walder went on to form Integrated DNA Technologies (IDT), who, in 1988, collaborated with Biosearch to determine which 2'-hydroxyl protecting scheme would work best. IDT focused on the groups cleaved with acid, while Biosearch focused on silyl protection chemistry. That head-to-head competition was won by Biosearch with their work on the *tert*-butyldimethylsilyl (TBDMS) group, which is still the most commonly used 2'-hydroxyl protecting group to this day.

Although procedures for the preparation of 2'-silyl phosphoramidites and 2'-deprotection of synthesized oligonucleotides initially described by Ogilvie were not perfect, it was satisfactory for the main application of the time, which was to provide a limited number of RNA targets to be used for the initial biochemical studies of antisense DNA oligonucleotides. A great number of publications appeared in the 1990s that described their use as targets, mostly by the leaders of the antisense industry: Isis Pharmaceuticals, Genta Inc, and Hybridon, Inc. Later developments in deprotection chemistry significantly improved the quality of RNA synthesis using silyl-protected nucleosides.^[74]

In the late 1980s and early 1990s, RNA chemistry also played a role in the development of a diagnostic assay that used a chimeric DNA–RNA–DNA oligonucleotide as the substrate for cleavage by RNase H.^[75] This reaction was cyclic and therefore created an amplification scheme whereby the signal was amplified through direct interaction of the probe with the target, but the target itself is not amplified, as is the case in PCR (Figure 7). The assay was conducted at a temperature where the oligonucleotide probe would bind to the target, be cleaved in half by the enzyme, the resulting pieces dissociated from the target, and the process repeating itself with a new oligonucleotide probe. It had the potential to be an isothermal amplification scheme, but was never reduced to practice as a viable alternative to PCR.

3.2.2. Ribozymes

The first significant new challenge to the RNA synthetic chemist commenced after the discovery of ribozymes by the groups of Altman and Cech.^[76] When it was discovered that RNA formed structures in nature that had enzyme-like qualities, such as sequence-specific cleavage of targeted nucleic acids, a number of labs and companies quickly exploited this knowledge.^[77] They produced synthetic RNA oligonucleotides that folded into “hammerhead” structures and acted as sequence-specific nucleases^[76c,78] (Figure 8). These somewhat longer oligomers were perhaps the first therapeutic drug candidates comprised entirely of RNA.^[79] The 35- to 45mer oligonucleotides taxed the current methodology, especially because the therapeutic applications required RNA modifications to afford nuclease stability of the ribozymes against cellular nucleases. The difficulties in manufacturing modified

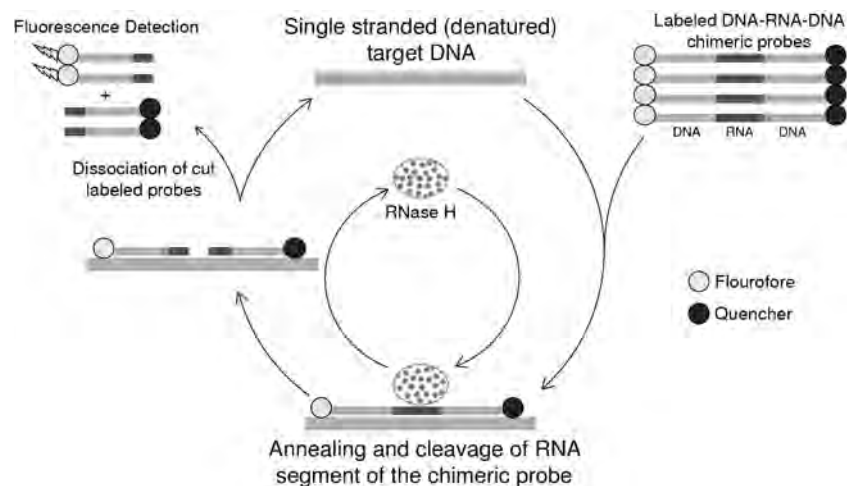


Figure 7. General scheme of signal amplification using cycling probe technology (CPT).

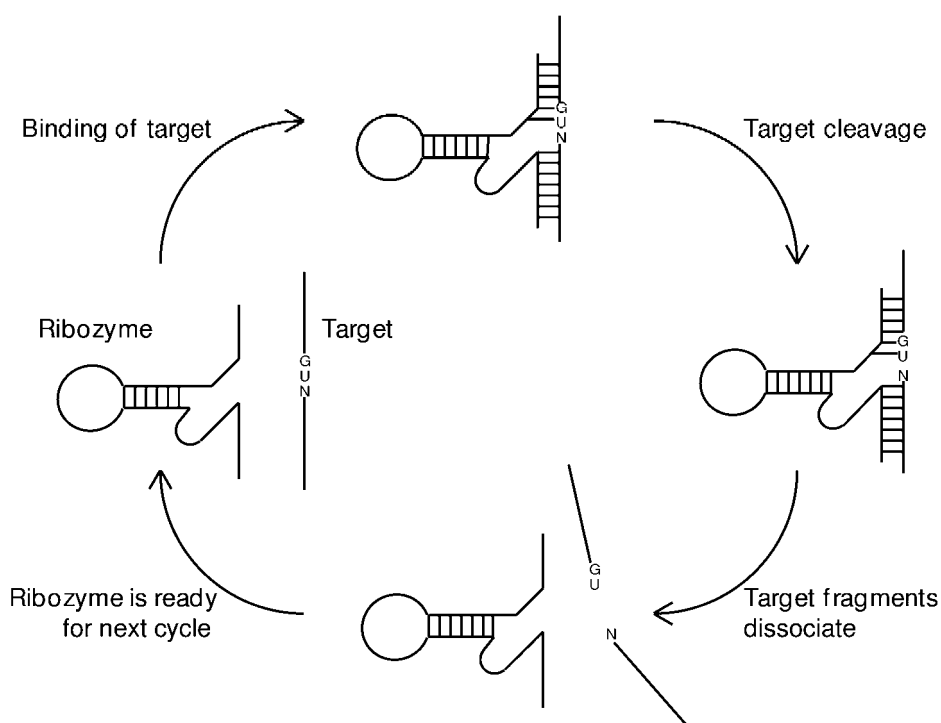


Figure 8. General scheme of target cleavage with a ribozyme.

ribozymes accompanied with the lack of clear clinical success in early trials^[80] led to the eventual downfall of the technology as a viable therapeutic option.

However, this field of study did yield one innovative turn in the synthesis of RNA oligonucleotides. A University of Colorado professor, Uhlenbeck, investigated the use of T7 polymerase to manufacture relatively short RNA oligonucleotides on larger scales through transcription.^[81] His detailed study of how T7 polymerase worked and how to use it to prepare large quantities of RNA was seminal. One significant drawback to this method is the

fact that sugar- and base-modified nucleotide 5'-triphosphates are not incorporated very well by natural T7 polymerase, limiting this procedure largely to the synthesis of unmodified RNA.

3.2.3. siRNA and MicroRNA

The next finding in the RNA world prompted a great deal of interest in synthetic RNAs that mimicked natural short interfering RNA (siRNA). The discovery of the ability of siRNA to cleave an RNA target in a sequence

specific manner was so significant that a Nobel prize was awarded to the discoverers, Fire and Mello.^[82,83] A great deal of research focus and money were invested in what was initially considered to be the answer to all of the woes of antisense DNA technology: it was thought that siRNA was much more active than antisense DNA; thus curtailing cost through a reduction in materials—the duplexes were sufficiently nuclease resistant; therefore, no modifications were needed to stabilize the oligonucleotide complex, and finally, these molecules could be delivered naked; thus eliminating the delivery problem.^[83] However, later studies demonstrated that nuclease resistance and delivery issues were still critical obstacles for the development of therapeutic siRNA,^[84] just as they were hurdles for the development of therapeutic antisense compounds. Still, from the point of instigating innovation in science, siRNA research helped drive oligoribonucleotide chemistry forward.

Chemically, the first problem that required a solution was the need for rapid and fairly inexpensive synthesis of large numbers of unmodified siRNA duplexes to silence genes. The main application was target validation. Although most firms licensed to produce siRNA for that purpose used traditional methods combined with more efficient, automated instrumentation and chemistry, one firm did offer an alternative. Dharmacon Inc., was the commercial entity formed to exploit 2'-ACE technology for the synthesis of RNA.^[85]

Because it is essential that siRNA constructs for therapeutic purposes are stable against nucleases and efficiently delivered *in vivo*, a large body of work ensued to overcome these problems. Most of the proposed solutions came directly from the antisense playbook, such as the incorporation of modified bases and phosphorothioate linkages to impart nuclease resistance and addition of cholesterol, lipids, and other conjugates that were found to largely direct traffic to the liver.^[86] One noted difference between the modification of RNA and DNA was the inability to use the neutral methylphosphonate backbone with RNA. The 2'-hydroxyl rapidly degrades the methylphosphonate linkages (R. I. Hogrefe, unpublished data, 1991).

Modifications to the RNA that were needed to obtain a therapeutically viable siRNA molecule were costly. Firms such as siRNA Therapeutics Inc. and Alnylam Pharmaceuticals expended a great deal of effort arriving at constructs that showed promise, but were ultimately costly to manufacture. As with antisense oligonucleotides, much work was placed in scaling up the existing synthesis technologies to reduce cost as much as possible. Many of the advancements discovered for scaling up DNA synthesis were, of course, applicable to RNA synthesis. Because these oligonucleotides were fairly short, generally 19–25 bases long, the length was not a challenge for current methodology. The largest reduction in cost was mostly in the price of the RNA phosphoramidite itself, which was

driven down due to the increase in manufacturing scales exactly.

MicroRNA (miRNA) is closely related to siRNA and mechanistically they come from the same general class of molecules that control the processing of genetic information.^[87] Exactly how they exert that control remains elusive, since the complex biology of RNA transcriptional control by other RNA is slowly being understood. Year by year, new revelations regarding the role of miRNA in the living cell make our current understandings seem almost elementary. They are similar to siRNA from a synthetic standpoint and all of the improvements made for siRNA synthesis were applicable, of course, to the synthesis of miRNA. Therapeutically, miRNA is also confronted with the same hurdles as siRNA and corresponding solutions are being used, such as nucleoside base and sugar-phosphate backbone modifications, as well as delivery vehicles.

3.2.4. RNA Aptamers

The most recent challenge to RNA chemical synthesis comes from the advent of RNA aptamers, which are nucleic acid motifs that bind with high affinity and specificity to target molecules through non-standard interactions^[88] (Figure 9). The combination of length, strong sec-

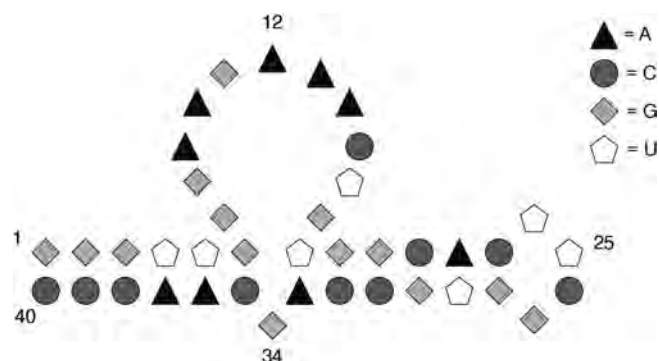


Figure 9. Secondary structure of an AMP-binding aptamer.

ondary and tertiary structures, and modifications make these oligonucleotides particularly difficult to prepare. To date, there have not been any reported innovations to RNA synthetic chemistry that address this particular problem. In many cases, the length of the aptamer is shortened from the original motif to identify a truncated version that is more easily synthesized, yet still retains the desired properties. This has been successful in a number of cases,^[89] but it is an expensive endeavor that may not always succeed.

Besides the inherent complexity of making a long RNA with secondary structure, the 2'-deoxy-2'-fluoro modifications commonly used to prepare nuclease-resistant aptamers introduce an additional problem. Our laboratory

has found that the 2'-deoxy-2'-fluoro nucleoside is sensitive to desilylating reagents and will undergo chemical modification (D. Combs, personal communication, February 21, 2013), thereby substantially lowering the yield of the desired product. (A search of the literature has not revealed a published resolution to this problem.) This issue needs to be addressed to make compounds containing 2'-fluoronucleosides viable candidates for therapeutic use. Although small-scale synthesis of these compounds may be reasonable for research use, the large scales needed for clinical trial and beyond become extremely expensive. An innovative solution to this problem is required. Perhaps a return to acid-labile protecting groups is the answer.

One such direction being explored is a return to the use of enzymes to produce RNA oligonucleotides, both at research and clinical scales. Recall that the use of T7 RNA polymerase to produce ribozymes at reasonable scales was explored in the past. Unfortunately, natural T7 polymerase does not incorporate 2'-modified nucleoside 5'-triphosphates very well, which is a critical component of many aptamers designed for therapeutic use.

3.2.5. mRNA and lncRNA

Although messenger RNA (mRNA) is generally not considered to be a synthetic chemical target, it warrants mention because it requires the same enzymes as those described above and it is becoming perhaps the next step forward in synthetic RNA products readily available to the researcher. A small number of firms are starting to sell modified and unmodified custom mRNA, which is only accessible through enzymatic synthesis. These compounds are commonly in excess of 1000 bases long, and sometimes surpass 3000 bases. Synthetic mRNA has been used to study the effect of introducing exogenous RNA to cells,^[90] as therapeutic agents,^[91] and to produce pluripotent stem cells from adult skin cells.^[92]

These applications require modified mRNA to reduce the immune response.^[90,93] Currently, these modifications are limited to those readily accepted for transcription by T7 polymerase and then readily translated in the cell. Mutated enzymes can also be applied to the synthesis of modified mRNA as well as aptamers. In fact, the ability to incorporate 2'-modified nucleoside 5'-triphosphates may be just as critical for the success of synthetic mRNA as a general tool as it is for aptamers.

Enzymatic methods can also be used to make long RNA in general. Recently, there was significant progress in the chemical synthesis of RNAs up to 110mer long;^[94] however, the need for very long RNA molecules (200mer and longer) still remains unmet due to limitations of traditional phosphoramidite RNA chemistry. While these long RNAs cannot be synthesized chemically, they can be prepared by *in vitro* transcription of DNA template (plasmid or PCR amplicon) using nucleoside 5'-triphosphates

and a DNA-dependent RNA polymerase, most often T7 or SP6 RNA polymerase. If RNA synthesis includes a 5'-(7-methylguanosine)-cap structure and a 3'-poly(A) tail, it can be used for protein expression.

Currently, there are commonly available kits for laboratory synthesis of up to milligram quantities of RNA. The advantage of RNA synthesized enzymatically from natural nucleoside triphosphates (NTPs) is the absence of unwanted modifications that are often present in chemically synthesized RNA oligonucleotides. Enzymatic synthesis does not require specific chemicals and solvents and fully fits into the concept of "green chemistry". However, T7 RNA polymerase adds one or more non-templated nucleotides at the 3'-end of the nascent RNA transcript, producing fragments that are longer than expected. These N+1 and N+2 products can be produced in equimolar or higher amounts than the desired product.^[95]

Another disadvantage of natural RNA (RNA containing only natural nucleoside units), is their susceptibility to cleavage by certain enzymes, to chemical conditions (such as high pH), or some metal ions.^[96] Additionally, natural RNAs, especially those with secondary structure, induce an innate immune response. Therefore, a number of modifications to RNAs were tested to improve *in vivo* stability and immunological properties of RNA molecules.^[97] It was found that T7 RNA polymerase (and other RNA and DNA polymerases) could accept a limited number of modified NTPs as substrates; thus allowing the preparation of partially modified mRNAs.^[98] To improve substrate properties of RNA polymerase towards modified NTPs, mutated versions of T7 RNA polymerase were constructed,^[98,99] which could readily accept modified NTPs.

Recently, a mutated DNA polymerase from *Thermococcus gorgonarius* was described that could perform the synthesis of RNAs up to 1.7 kb long and fully substituted with pseudouridine, 5-methyl-C-, 2'-fluoro-, or 2'-azido-modified NTPs primed from a wide range of primer chemistries comprising DNA, RNA, locked nucleic acid (LNA), or 2'-O-methyl-DNA.^[100] While the enzymatic approach may be the best solution for the synthesis of long RNA to date, it does not meet the need to be able to incorporate modified ribonucleotides in specific locations. For this, novel chemistry is still needed.

3.3. DNA Oligonucleotides in the Future: Unmet Needs

While making modified long RNA may be the biggest challenge facing oligonucleotide chemists today, there are a number of applications that require DNA oligonucleotides with modifications that are difficult to make, or require extremely high purity that go beyond what is normally available on the market. As with RNA, we look at the requirements and unmet needs of DNA oligonucleotide synthesis from an applications point of view.

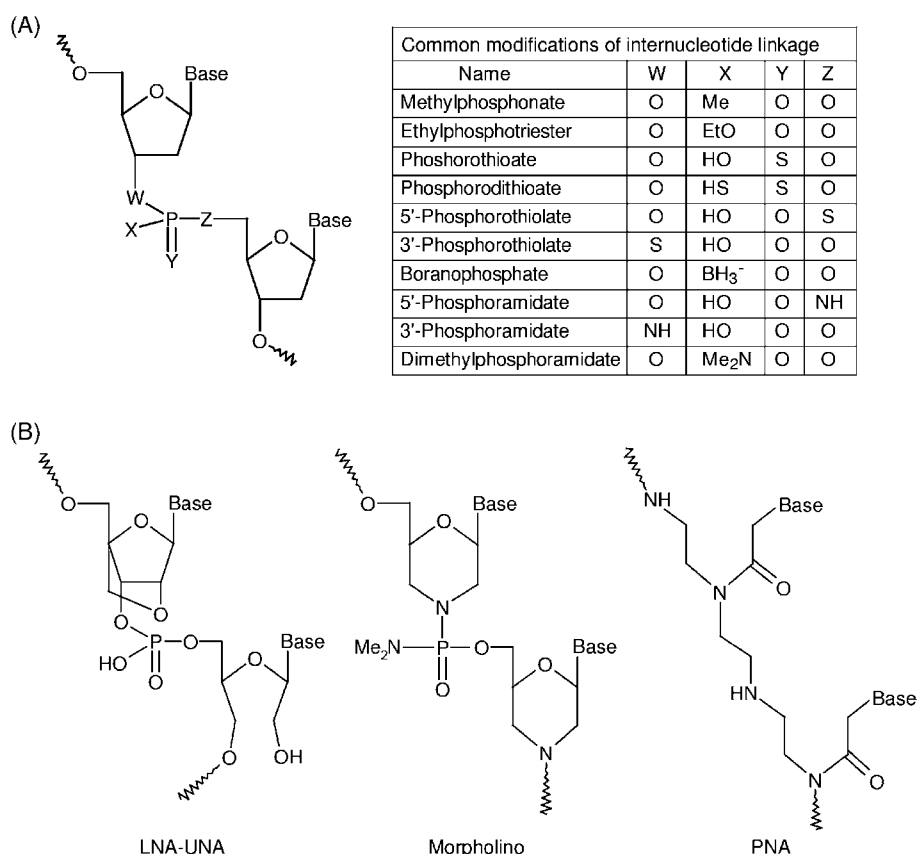


Figure 10. Structures of several common modifications of internucleotide linkage units used in modified oligonucleotides (A). Structural units of locked nucleic acid/unlocked nucleic acid (LNA/UNA), morpholino, and peptide nucleic acid (PNA) oligonucleotide analogues (B).

3.3.1. Modified Oligonucleotides and Their Analogues: A New Generation of Change

DNA oligonucleotides containing modified sugars, internucleotide linkages, and nucleoside bases have a long history in oligonucleotide chemistry (Figure 10). Oligonucleotides containing modifications such as phosphorothioates,^[101] alkylphosphotriesters,^[102] and methylphosphonates^[103] have been reported as early as the 1960s and 1970s and were the basis for most of the antisense oligonucleotides studied in the 1990s; a number of which are still in clinical trials, such as Genta's Genasense[®], which is a phosphorothioate-modified oligonucleotide targeted against BCL-2.^[89e,104] Few have made it to market, the most notable being Vitravene[®] from Isis and, very recently, Food and Drug Administration (FDA)-approved Kynamro[™], which is another antisense drug from Isis.

As with RNA, the impetus for the current need for novel DNA modifications has been the emergence of new applications. Many applications need an adjustment or change of physical, chemical, or biological properties of the oligonucleotides to fit specific requirements. Since the number and complexity of such applications is constantly increasing, the need for more sophisticated oligonucleotides is growing in parallel.

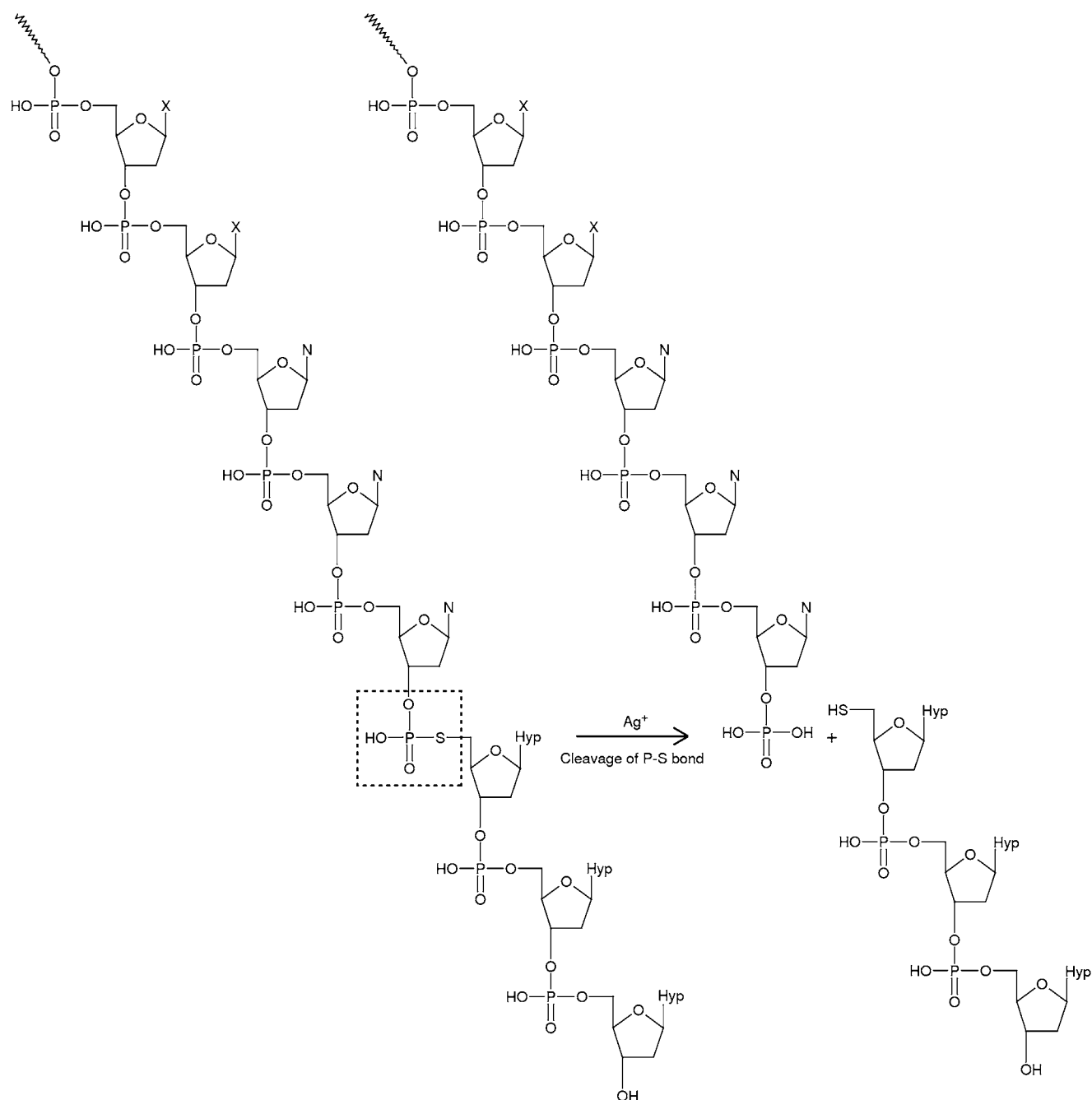
Over the past few decades, methods have been identified to finely tune the properties of oligonucleotides. This list is long and includes hybridization affinity to target sequences, stability against nucleases, conformational attributes, hydrophobicity, charge, spectral properties, and delivery uptake properties.^[105] Many of the above properties were attained by conjugating oligonucleotides to a seemingly limitless number of molecules carrying specific functionalities (e.g., biotin, fluorescent dyes, and antibiotics).^[106]

A large number of new modifications synthesized to achieve various physical, chemical, or biological tasks have been introduced in the last fifteen years or so. Among these are several oligonucleotide backbone (sugar and/or phosphate) modifications: the most well known are LNA,^[107] UNA,^[107d,e,108] morpholino,^[109] borano,^[110] amidophosphate,^[111] and phosphorodithioate^[112] derivatives. Also, there are numerous variants of oligonucleotide analogues with dephospho-internucleoside linkages,^[113] the most well-known example of which are PNAs.^[114] There are several comprehensive reviews that describe the synthesis, physicochemical and biological properties, and various applications of modified oligonucleotides and their analogues.^[105,106,115] Below we present two examples where oligonucleotide construct design

and nucleotide modifications were specifically adjusted for the application.

SOLiD™, one of the next-generation sequencing platforms currently on the market, is an example of an application that requires highly modified oligonucleotides.^[116] The SOLiD™ process for sequencing is a fairly complicated process of ligation and amplification steps that is best described graphically. There is an excellent video on YouTube,^[117] which is far better than a written description

of the process. The key oligonucleotide component of the SOLiD platform is a set of fluorescently labeled 8mer oligodeoxyribonucleotide probes. The first two positions of each probe are comprised of a dinucleotide sequence specific to the fluorophore. Nucleotides three to five are randomized nucleotides that are used to increase the sequence complexity of the probe. The last nucleotide units, six to eight, are made up of three universal nucleosides (2'-deoxyinosines; dI₃) and attached to the rest of the



Scheme 7. Cleavage of a phosphorothiolate linkage in an octanucleotide probe is a principal chemical step in Life Technologies SOLiD sequencing platform. X = interrogated base, N = randomer base, Hyp = hypoxanthine (base of 2'-deoxyinosine nucleoside).

probe by a cleavable phosphorothiolate linkage. The fluorophore is attached to the end of the dI_3 sequence. After each successive round of ligation, a phosphorothiolate linkage of the ligated probe is cleaved (Scheme 7), with silver ions, releasing a dI_3 -fluorophore fragment and reducing the length of the ligated probe to five nucleotides, ready for the next round of ligation. This construct contains modified bases, a modified backbone (bridging the phosphorothioate), a random sequence, and a fluorophore. The oligonucleotide chemists involved spent several years optimizing the chemistry for commercialization.

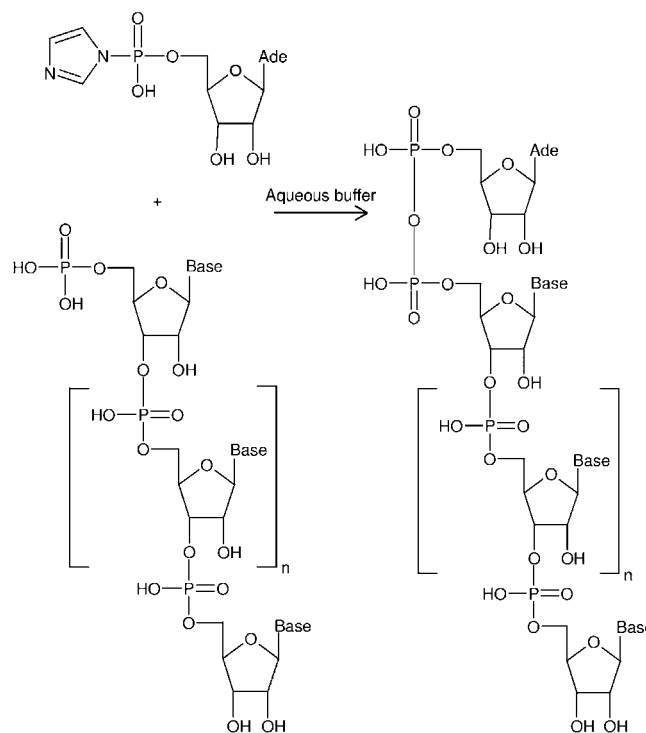
Another area where highly modified DNA oligonucleotides are currently being explored is the use of DNA aptamers for both therapeutic and diagnostic applications.^[88c,89d,118] Like RNA aptamers, these are often longer oligonucleotides and can contain multiple modifications. There are many more modifications available for the chemical synthesis of modified DNA aptamers than there are for the previously discussed RNA aptamers. Since DNA is a much easier synthetic target, many modified reagents exist that can be incorporated into the aptamer after the initial selection process to allow fine-tuning of the aptamer's properties.^[119] Also, many of the modifications, particularly those of the 5 position of pyrimidines and 7 or 8 positions of purines, can be directly incorporated during the selection process because DNA polymerases will generally tolerate those modifications.^[119,120]

Besides the commonly incorporated 2'-OMe- and 2'-F-modified nucleosides that are used to enhance the stability of the secondary structures and impart nuclease stability for therapeutic DNA aptamer candidates, many other modifications have been explored. SomaLogic Inc., one of the first firms formed to commercially exploit aptamers, has developed many different base analogues that offer a wide pool of different properties to choose from.^[121] Many of these modifications allow unique interactions with specific molecules, such as proteins, thereby producing much more potent aptamers. LNA-modified aptamers are also being explored as a way to produce novel structures with tighter binding characteristics.^[122]

Recent achievements in generating mutated nucleic acid polymerase constructs with a broad range for recognition of sugar-modified nucleoside 5'-triphosphate analogues allowed the successful transcription of DNA to complementary xeno nucleic acid analogues (XNAs) containing fully modified sugar residues, such as LNA, fluoroaribino nucleic acid (FANA), aribino nucleic acid (ANA), threose nucleic acid (TNA), hexitol nucleic acid (HNA), and cyclohexene (CeNA). Moreover, it was shown that these modified XNA polynucleotides could be transcribed back to the DNA strand with the original nucleotide sequence and with a very low mutation rate, demonstrating that "... heredity and evolution, two hallmarks of life, are not limited to DNA and RNA but are likely to be emergent properties of polymers capable of information storage."^[123]

3.3.2. Adenylated Oligos—Ligase Ready

A tool that is extremely valuable for current biological techniques is the ability to deep sequence using the latest generations of sequencing platforms. While most of the oligonucleotides needed for these platforms, such as standard primers, do not challenge the ability of oligonucleotide chemistry from a structural standpoint, there are several exceptions. One area of innovation has been in the preparation of 5'-adenylated oligonucleotides, used in ligation-based RNA library preparation common to most of the current sequencing methodologies^[124] (Scheme 8).



Scheme 8. Chemical synthesis of 5'-adenylated oligonucleotides required for adenosine triphosphate (ATP)-free ligation applications. Ade = adenine base.

By pre-adenylating the oligonucleotides, the addition of adapters can be introduced with high specificity onto RNA libraries. The use of pre-adenylated oligonucleotides is especially important where ligation bias is an issue, such as in miRNA sequencing.^[125]

There are two principle ways to make 5'-adenylated oligonucleotides: chemical and enzymatic. The chemical route includes the reaction of a 5'-phosphorylated oligonucleotide with a pre-activated form of adenosine-5'-monophosphate, such as the phosphoryl imidazolide, in solution^[124,126] or on a solid support.^[127] This approach can be applied to the synthesis of both RNA- and DNA-adenylated oligonucleotides. The downside of the chemical approach is an unavoidable purification step to separate

the adenylated product from the unreacted starting oligonucleotide. For short oligonucleotides, it can be accomplished by HPLC or polyacrylamide gel electrophoresis (PAGE); however, for longer oligonucleotides and, especially, polynucleotides the separation becomes problematic.

The enzymatic route relies on the ability of DNA or RNA ligases to transfer an adenosine monophosphate (AMP) residue from the activated enzyme to the 5'phosphate of an oligonucleotide.^[128,45a] This approach is convenient, since it does not require any specific reagents, except ATP ligase and the appropriate buffer. However, DNA ligase does require additional oligonucleotides for adenylation to occur: specifically, an oligonucleotide template and an acceptor (often 3'-modified).^[128a,129] While RNA ligase does not require a template for adenylation, to improve the reaction yield, a "blocking" oligonucleotide or, alternatively, a 3'-blocked donor oligonucleotide^[128b] should be used to reduce the formation of side products.^[124,130,131] Both approaches often suffer from incomplete adenylation.

Recently, a thermostable RNA ligase was introduced that could perform nearly quantitative adenylation of 3'-unprotected oligonucleotides in the presence of a high concentration of ATP, which blocked acceptor circularization and oligomerization.^[132]

3.3.3. 3'-Blockers—Enzyme Control

Another modification that is critical for oligonucleotides used in many next-generation sequencing library preparation schemes are 3'-blocked oligonucleotides that cannot be ligated or extended enzymatically, depending on the process. Mostly these are used to ensure that ligation of the adapter sequences occur in the orientation desired. The modification that is traditionally used to block the 3' terminus of the oligonucleotide for this purpose is a 2',3'-dideoxynucleoside, which is necessary for some applications, such as pyrophosphorolysis-activated polymerization (PAP).^[133]

At one time, all four of the 2',3'-dideoxy bases coupled to a solid support were available commercially. Over time, the commercially available supports have dwindled to one: 2',3'-dideoxycytidine (ddC). These bases are linked to the support through the exocyclic amine in the cases of ddA, ddC, and ddG and the O-4 position of ddT. Unfortunately, the support-bound reagents are fairly unstable, with the exception of ddC, which remains on the market. The main problem with the ddT support is the intrinsic instability of the ester linkage from the O4-position of thymidine to the solid support. The main problem with the purine supports is the extremely high rate of depurination of 2',3'-dideoxy purine nucleosides.^[134] Since the growing oligonucleotide chain is attached to the support through a nucleobase, each depurination event results in a considerable loss of oligonucleotide from the

support, releasing the elongating oligonucleotide into solution. The cytosine base forms a more stable glycosidic linkage with 2'-3'-dideoxy sugar than adenine or guanine, and therefore, is less susceptible to acid-catalyzed deglycosylation than the analogous purines. Therefore, it survived as a commercial product. The ddA and ddG supports were available from Glen Research and Peninsula Labs until about ten years ago.

The other foreseeable solution for preparation of 2',3'-dideoxy-terminated oligonucleotides is a reverse 5'-3' oligonucleotide synthesis. In this case, 2',3'-dideoxynucleoside 5'-phosphoramidite can be added at the last coupling step to 3'-end of the synthesized oligonucleotide. The 5'-3' route is not a viable commercial alternative, due to low efficiency of the synthesis and to the high cost of nucleoside 5'-phosphoramidite monomers.

The need for these supports does exist, as evidenced by the large body of literature published using what supports were available at the time, but there does not appear to be a general solution for the chemistry problem to date. Given the increase in general interest in the PAP method, this is a major issue. A patent was recently applied for that offered a solution for the synthesis of support-bound ddT that may work. The inventors prepared a linkage to CPG through the N3 position of the thymine ring, which led to a reported loading of approximately $13 \mu\text{mol g}^{-1}$.^[135] The methods described for the loading of ddG and ddA on the support goes through the exocyclic amines, similar to the previously available supports, and thus, are still susceptible to depurination. There was no evidence at the time of this review that the assignee of the patent, Life Technologies, had plans to pursue the commercialization of this patent as a readily available reagent.

One reason there has not been a large amount of pressure to resolve this problem may be because most researchers and assay developers have another solution to the problem that is inexpensive and does exist on the market: support-bound propyl C3^[136] and amino linkers.^[137] Unless the assay being developed requires ddN at the terminus, such as with the PAP method, or is adversely affected by a 3'-terminal modifier on the oligonucleotide, these simple and cheap solutions are very effective at blocking enzymatic activity at the 3' terminus. Surprisingly, although this support has been commercially available since the 1990s and is well described in the literature for both therapeutic applications and assay development,^[138] there still seems to be a number of researchers that are unaware of this effective blocking reagent. Another effective solution, although more expensive than the propyl or amino linkers, is an inverted base (3'-3' coupled) at the 3' terminus.^[139]

3.3.4. High-Quality DNA—A Change in Paradigms

As mentioned in the Introduction, the driving force for innovation in the chemical synthesis of oligonucleotides in the past was chiefly the need to make them as cheap as possible and deliver them within a day or two. Oligonucleotide synthesis scales have dropped to a starting scale of as little as 5 nmol. Synthesis methods were developed to decrease the cycle time, significantly reduce reagent usage, and simplify purification. These modifications to the original protocol generally led to a decrease in overall quality. One example of this is the replacement of dichloroacetic acid (DCA) for the less expensive and more reactive trichloroacetic acid (TCA). TCA removes the trityl group from the 5'-hydroxyl group much quicker than DCA, but also exacerbates the problem of depurination, leading to more abasic sites, especially with longer oligonucleotides.

There has been an “underground” of molecular biologists and assay developers that understood the need for high-quality DNA oligonucleotides to obtain the highest quality experimental results.^[64,89e,140] However, the problems with the chemical synthesis of oligonucleotides are becoming even more critical as more applications are being developed that require very high quality oligonucleotides^[141] that are over 70 bases in length. In particular, the process of gene synthesis would be greatly improved if DNA oligonucleotides of 100 bases or longer could be made efficiently with fewer modifications. The effort that goes into the selection of genes containing the correct sequence is costly and time consuming.^[142]

PCR is often seen as a very rugged technique, where the main concerns are target clean up and primer selection. The quality of the primers themselves was not seen as important, and was the main reason that oligonucleotide chemistry focused on inexpensive, fast turnaround methodology to support research that required small amounts of a large number of primers to optimize PCR performance. In general, this is true. Most PCR applications are not overly rigorous and can allow for the use of crude, low-quality primers. As long as oligonucleotides with the proper sequence are present, the amplification typically moves forward as desired, since the primer is in large excess over the target. There is, however, mention in the literature about the reproducibility of assays from primer batch to primer batch,^[143] but the quality was not addressed as a general topic.

Moreover, there are techniques where the quality of the primers appears to be crucial. This is particularly true when one is trying to detect subtle differences in target sequences, such as SNP detection. Lot-to-lot variations in the quality of the primers can lead to significant differences in the output of PCR-based assays, which are required to be rigorous. For example, the forensics industry, where reproducibility is critical, is starting to realize that their results improve with the use of high-quality, highly puri-

fied primers.^[144] In fact, it is likely that all next-generation diagnostics and sequencing methods will require very high purity primers to achieve the level of sensitivity and reproducibility required.

Another interesting application, which depends on a precise and efficient synthesis of very long (>100mer) oligodeoxynucleotides, is the use of DNA molecules as an information depository. The first successful attempt of using DNA molecules as an alternative way to store non-biological information was demonstrated in late 1980s.^[145] However, only recently the real potential of DNA to store a large volume of readable and copyable information became a real prospective for the future.^[146] This achievement became possible in part due to the latest improvements in the quality of a multi-parallel DNA synthesis supported by an Agilent solid-phase synthesis platform.

Difficulties in the synthesis of high-quality oligonucleotides, especially long ones, are caused by several factors. For example, the coupling efficiency of the phosphoramidites needs to be very high. A small decrease in coupling efficiency can mean the difference between success and failure. For instance, a 100mer made with phosphoramidites with an average coupling efficiency of 99.5% will have the theoretical yield of 0.995^[99] or 61%, whereas if the coupling efficiency dropped slightly to 99%, the overall yield would be 37%. Drop to 98% and the theoretical yield is down to 13% (Figure 11). Maintaining very high coupling efficiency throughout the synthesis of a 100mer is not trivial. The sequence itself can directly affect the coupling efficiency as well by causing significant decreases in individual couplings. The latter does not significantly affect the average coupling efficiency, but affects the quality of the crude product mixture tremendously. The capping, detritylation, and oxidation steps all have to go nearly quantitatively as well. A slight decrease in efficiency in any of those steps will lead to truncated sequences

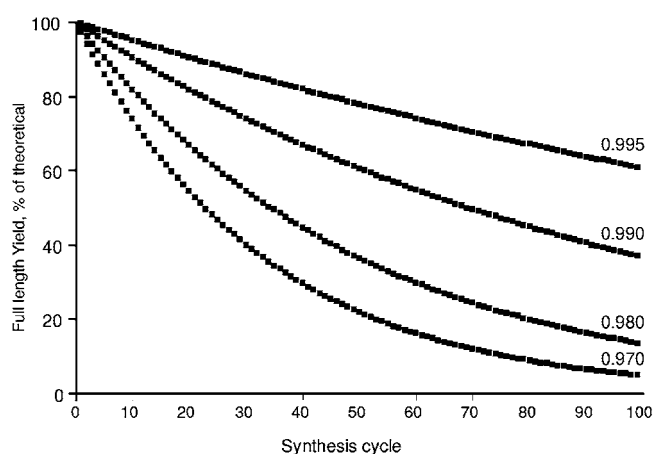


Figure 11. Dependence of the final yield (%) of the full-length oligonucleotide on the efficiency of oligonucleotide synthesis. The number represents an average efficiency of each synthesis cycle.

and heterogeneous admixtures that are difficult to resolve.

Additionally, the purification of these long oligonucleotides is also problematic. HPLC methods are generally considered to be effective to a length of 50 bases. PAGE can be useful for longer lengths, but the method is not cost effective for the purification of more than a few milligrams of material. Even the analysis of long oligonucleotides is difficult, since the crude product is typically a heterogeneous mixture of desired product along with truncated and base-modified sequences. A recent book was published that described many of the advances in oligonucleotide purification and analysis techniques.^[147]

Perhaps the largest obstacle to the synthesis of long DNA is the depurination of adenosine bases under the acidic conditions used for detritylation.^[64] Although the amount of depurination is small at each cycle, this builds tremendously over the synthesis of a long oligonucleotide to the point where most of the oligonucleotides in a crude mixture contain at least one depurinated site. Unfortunately, these sites are not as efficiently cleaved during deprotection of the oligonucleotides with base, forming easily removed truncated products, as previously reported.^[148] This problem affects the gene synthesis industry tremendously, whereby abasic sites lead to sequence mutations in the gene being prepared. Multiple clones must be picked and sequenced until one is found with the proper sequence.^[142] This problem has been addressed for the synthesis of microarrays through the optimization of the detritylation step, mainly by ensuring that the acid is quenched as soon as the detritylation step is finished, carefully ensuring that contact with acid is minimized.^[64]

A great deal of work has to be done to allow the chemical synthesis of very long DNA oligonucleotides, which begs the question regarding the use of enzymes for this. As mentioned in earlier sections, the use of enzymes to produce oligonucleotides has a long history. The recent need for long oligonucleotides had led some to reopen this line of inquiry for the synthesis of long DNA and RNA oligonucleotides. A major advantage of the enzymatic approach is the high chemical integrity of the final product. The bases are not modified and the resulting sequence is error free. However, as always, the major limitation of the approach is the inability to place single modifications in specific positions of the polynucleotide chain.

4. Summary and Outlook

The need for novel methods of preparing long modified DNA and RNA in higher quality is apparent. The requirements of the molecular and cellular biologists are once again outstripping the current ability of the oligonucleotide chemist to supply the required reagents. Many

researchers are being stymied by an inability to obtain what is necessary for their experiments because of either technical barriers or excessively high costs.

These needs are most apparent for the synthesis of long RNA, which is becoming more important as the biology of RNA, particularly lncRNA and miRNA, is being elucidated. The ability to selectively modify lncRNA and miRNA will be crucial for the understanding of molecular interactions with other components of the cell, as well as the development of diagnostic and therapeutic reagents. DNA oligonucleotides that are longer, purer, and containing only desired and no undesired modifications are also crucial for current research applications. Although enzymatic synthesis may be the solution for some of these needs, chemical synthesis will eventually need to catch up because the only way to incorporate specific point modifications in an oligonucleotide is to chemically synthesize that molecule. We oligonucleotide chemists are again confronted with the same dilemma we resolved once before—how to make and then specifically modify the biomacromolecules needed to continue our study of nucleic acids and how they function to help create life.

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