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Manufacture of therapeutic oligonucleotides: Development of new reagents and processes

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The optimization of synthesis, purification process parameters and development of new reagents have enabled successful large-scale manufacture of several therapeutic grade oligonucleotides at Avecia. The development of new reagents and processes are described in this communication.

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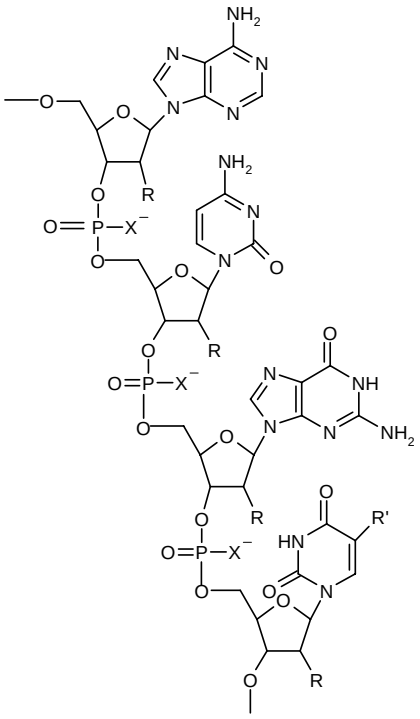
The solid phase synthesis of oligonucleotides at smaller scales is well understood and the processes at this scale do not impose any impact on economic, environmental or safety considerations. However, apart from economic consideration, significant challenges still exist for large-scale manufacture. More than a decade's effort and continuing searches for therapeutic agents based on antisense, ribozyme, immunostimulatory, aptamer, decoy and recent RNAi technologies have provided impetus for the large-scale manufacture of therapeutic grade oligonucleotides. Currently there are more than 200 oligonucleotides under preclinical and clinical investigations[1] for various diseases like cancers, autoimmune, asthma, and allergy. The clinical investigations require synthetic olignucleotides ranging from a few-grams to several kilograms. The synthetic oligonucleotides under investigations are classified as phosphorothioate oligonucleotides (first generation modified), chimeric oligonucleotides (second generation modified oligonucleotides where sugar and/or heterocyclic base and internucleotidic linkages are modified), short dsDNA (double stranded deoxy nucleic acids), dsRNA (double stranded ribonucleic acids) as well as standard phosphate diesters for aptamer technology. The structure of these various oligonucleotides are presented in **Figure 1**.

Oligonucleotide syntheses, irrespective of the scale or type (DNA, RNA, aptamer etc.) are performed using β-cyanoethyl-phosphoramidite chemistry using solid support[2,3] in an automated synthesizer[4]. The synthesis proceeds from 3′-end to 5′-end of the sequence. The 3′-end nucleoside is covalently attached to the solid support in such manner that, at the end of synthesis, the covalent linkage can be removed easily. The chemistry utilized and steps involved in the synthesis of oligonucleotides are represented in the **Scheme I** and **Figures 2**, **3** and **4**. The chemical reactions in solid support mediated synthesis are almost quantitative, driven by the use of excess reagents. In smaller scales of synthesis, the use of excess amount of reagents, chemicals and solvent may not have significant impact on costs, environmental safety and availability of raw materials supply. However, at larger scales (100 to 500 mmole) all of these issues become major hurdles for the development of oligonucleotide as a viable drug product. The challenges for large-scale oligonucleotide synthesis can be outlined as:

(i) Quality of the material
(ii) Scale-up of small-scale R&D/pre-clinical manufacturing processes
(iii) Equipment and reagents

List of Abbreviations: DsDNA=Double stranded deoxynucleic acids; dsRNA=Double stranded ribonucleic acids; DMT=Dimethoxytrityl; DCA=Dichloroacetic acid; TCA=Trichloroacetic acid; P=Phosphorus; G=Guanosine; RP-HPLC=Reverse phase-High performance liquid chromatography; SMI=Saccharin-methyl imidazole; PADS=Phenyl acetyl disulfide; XHS=Xanthane hydride solution; UF=Ultra filtration.



Adenosine

Cytidine

Guanosine

Thymidine when R' =CH_3 &
Uridine when R' = H

Natural Oligonucleotides:

a. Oligodeoxynucleotide (DNA) when R = H, R' = CH_3 and X = O
b. Oligoribonucleotides (RNA) when R = OH, R' = H and X = O

First Generation Modified Olignucleotides:

a. Phosphorothioate Oligodeoxynucleosides (S-DNA) when R = H, R' = CH_3 & X = S
b. Oligodeoxynucleotide with mixed phosphate & phosphorothioate diseter linkages

Second generation of Modified Oligonucleotides:

a. Chimeric Oligonucleotides; where some of ribo-sugar is modified (R = F', OCH_3, $OCH_2CH_2OCH_3$ or NH_2) and may have phosphate and phosphorothioate diester linkages
b. Chimeric Oligonucleotides where some of the heterocyclic bases (eg. 5-methylcytosine) as well as ribo-sugar are modified. In addition to these may have phosphate and phosphorothioate diesters linkages

Figure 1—Truncated structure of natural and modified oligonucleotides

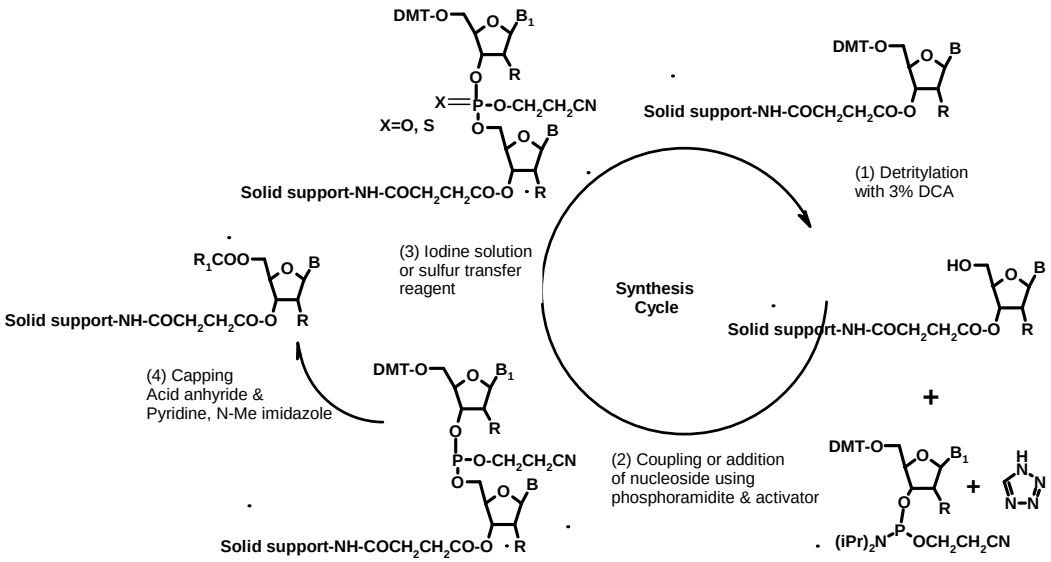
(iv) Cost Reduction
(v) Safety, Health and Environmental issues, primarily minimization of hazardous chemicals use and waste generation

The manufacturing process consists of two parts; the steps involved in synthesis of the oligonucleotide and removal of protecting groups as the first part, and purification and isolation as the second part.

Results and Discussion

Synthesis Strategy and Steps

(i) Detritylation or removal of DMT (dimethoxytrityl) group from 5′-terminal hydroxyl of ribo-sugar with DCA (dichloroacetic acid) or TCA (trichloroacetic acid) solution.
(ii) Coupling or addition of nucleoside for chain elongation using nucleoside phosphoramidite and activator mixture; activator being tetrazole solution.
(iii) Conversion of P(III) (phosphorus III) to P(V) (phosphorus V) stable linkage between two nucleosides. In phosphate diester, iodine solution containing certain percentage of water is used. For synthesis of phosphorothioates, various sulfurizing reagents are available.
(iv) Capping or blocking of free terminal hydroxyl groups on uncoupled oligonucleotide chains,



Scheme I—Chemical steps involved in oligonucleotide synthesis

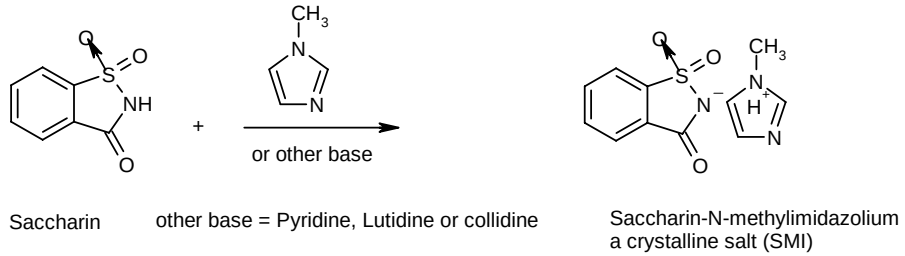


Figure 2—Synthesis of Saccharin based activators

with acetic anhydride in presence of tertiary heterocyclic base.

(v) Release of the synthesized oligonucleotide chain and removal of protecting groups with concentrated ammonium hydroxide. For oligonucleotides containing RNA bases, a second deprotection step is required to remove the silyl protecting groups on 2′-hydroxyl positions.

Generally, a large amount of 3% DCA/TCA solution in dichloromethane is used during the detritylation step. Dichloromethane is not an environment friendly solvent. Tetrazole has been activator of choice for coupling since introduction of phosphoramidite chemistry, however, recently has been classified as an explosive. The number of equivalents of reagents used for small to medium scale syntheses is generally high. For the synthesis of phosphorothioate oligonucleotides, Beaucage or other reagents are used for sulfurization. Beaucage reagent is not very stable, requires special handling and generates side products. Acetic anhydride is most commonly used for capping. In large-scale synthesis of G (guanonsine) rich sequences, it has been observed that some isobutyryl groups are replaced with acetyl groups as a result of *trans*-amidation during capping steps and under standard cleavage and deprotection condition acetyl group is not removed, creating additional impurities.

During cleavage and deprotection, phosphorus-protecting cyanoethyl group is released as acrylonitrile, which can react with heterocyclic bases creating additional impurities. Acrylonitrile has been found to modify Thymidine and Cytosine.

Purification strategy and steps

In contrast to synthesis, there are three distinct chromatographic approaches that can be adopted for purification of synthetic oligonucleotides. These chromatographic approaches are RP-HPLC (reverse phase high performance liquid chromatography),

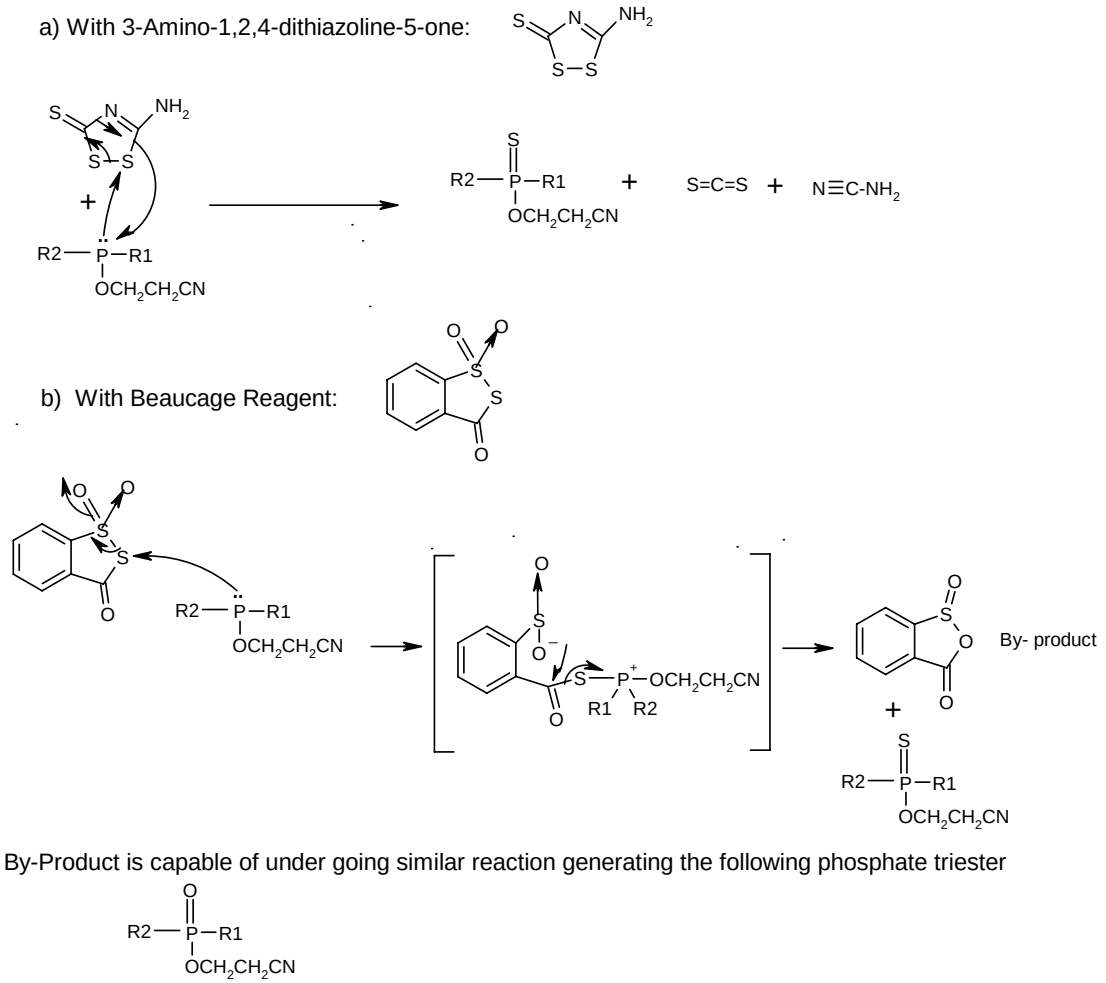


Figure 3—Mechanism of sulfur transfer reaction

Hydro phobic Interaction chromatography followed by Weak Anion exchange chromatography, and Strong Anion exchange chromatography. At the development stage of manufacture of synthetic oligonucleotide, these methods were utilized for purification. The steps involved in these different approaches are described below.

1. Purification by RP-HPLC using eluent that contains organic solvents
   a. Removal of ammonia by evaporation
   b. RP-High Pressure Chromatography
   c. Selection of Fractions
   d. Removal of Organic solvent
   e. Detritylation with acetic acid
   f. Removal of acetic acid
   g. Conversion to appropriate salt
   h. Ultra filtration
   i. Lyophilization
   j. Isolation of product
2. Purification by Hydrophobic Interaction Chromatography followed by Weak Anion Exchange Chromatography
   a. Capture of DMT-on material by hydrophobic interaction
   b. Elution of DMT-on material
   c. Adjustment of concentration oligonucleotide and salt
   d. Detrityaltion with acetic acid
   e. Loading onto weak anion exchange column
   f. Elution of the product by chromatography
   g. Selection of fractions
   h. Ultra filtration

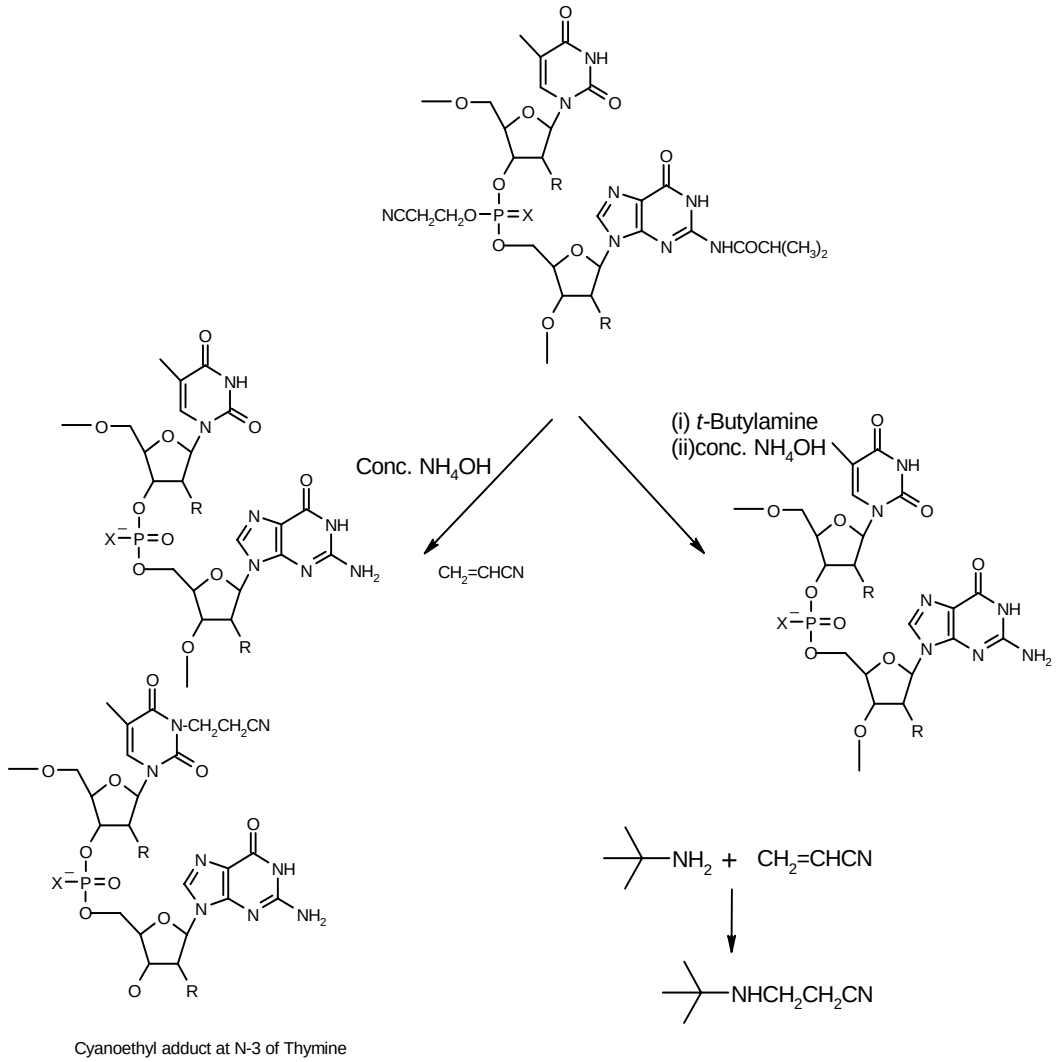


Figure 4—Preventing formation of cyanoethyl adduct of thymine

i. Lyophilization
j. Isolation of product

(This is a two-step chromatography process)

3. Purification by Strong Anion Exchange Chromatography
   a. Loading of DMT-on crude onto strong anion exchange column
   b. Removal of DMT-off failure sequences with salt solution buffer B (25-40%B) at *p*H ~12
   c. Removal of salt by washing with water or buffer A (no salt)
   d. Detritylation, on column removal of DMT-group from the oligonucleotide
   e. Elution of the desired oligonucleotide
   f. Selection of fraction
   g. Ultra filtration
   h. Lyophilization
   i. Isolation of product

The two most commonly utilized purification processes are RP-HPLC and Strong Anion Exchange Chromatography. The RP-HPLC purification method requires the use of large amounts of organic solvent, raising safety concerns, and the use of large quantities of the media due to low loading capacity, raising the cost. The important and primary considerations for purification at larger scales are cost, safety and throughput. Strong Anion Exchange Chromatography addresses some of these considerations. Strong Anion Exchange Chromatography allows higher throughput, predominantly utilizes aqueous salt solutions as eluents, and scales up easily. The process adopted at

Avecia Biotechnology for purification at large-scale is based on Strong Anion Exchange Chromatography.

Development of New Reagents and Processes

The solutions to some of these challenges have been achieved by developing new reagents and processes at Avecia. In the past several years our development activities have been focused on reagents and processes that allow the use of safe and environmentally-friendly chemicals, minimize toxic waste generation and manufacture economically so that oligonucleotides can be viable drug products. These activities have been categorized as following:

(i) Replacement of dichloromethane with toluene in dichloroacetic acid solution: To minimize Health and Environment concerns and reduce hazardous waste generation.

(ii) Development of SMI (Saccharin methylimidazole) to replace tetrazole as activator: To reduce safety and supply concerns, as tetrazole has been classified as Explosive substance.

(iii) Minimization of impurities by using alternate reagents: 3-Amino-1,2,4-dithiazoline-5-one, *tert*-Butylamine and isobutyric anhydride to improve quality and reduce the production cost.

(iv) Use of strong Anion Exchange Chromatography for higher throughput and to minimize hazardous waste generation and operator safety concerns.

Solid support: Amino derivatized beads made out of either Controlled Pore Glass or Polystyrene, onto which the first nucleoside from 3′-end is covalently attached. Nucleoside Phosphoramidites are the building blocks, and activator is generally a mildly acidic compound to facilitate chain elongation.

Iodine solution: 20 to 50 mM Iodine solution in pyridine containing 4-10% water.

Sulfur Transfer Reagent: There are several reagents, including Beaucage, PADS (phenyl acetyl disulfide) and XHS (xanthane hydride). Solutions are made in acetonitrile, acetonitrile-lutidine/picoline mixture and or pyridine.

Acid Anhydride: Acetic anhydride is commonly used together with N-methylimidazole and pyridine in acetonitrile.

Replacement of dichloromethane in DCA solution

A large excess dichloroacetic acid solution is generally required for removal of 5′-terminal dimethoxytrityl group. The amount could be 1000 to 1500 L of 3% DCA solution in dichloromethane per synthesis at scales higher than 100 mmoles. The annual consumption of dichloromethane could be more than 200,000 L. The use of such a large volume of chlorinated solvent would not only expose the personnel to potential safety and health hazards but would also generate large volume of hazardous waste. In order to minimize this, Avecia replaced dichloromethane with the more environmentally-friendly solvent toluene[5]. The use of 3% DCA in toluene has not compromised quality of product nor introduced any new impurity.

Development of non-explosive activator SMI to replace tetrazole

Tetrazole has been used as an activator since the introduction of phosphoramidite chemistry in the 1980s[2,3]. This reagent has been most popular due to mild acidity sufficient to activate phosphroamidite for efficient coupling without causing any side reaction. Tetrazole is still used for small-scale synthesis. Since regulatory authorities in USA and Europe classified solid tetrazole as an explosive, the search for alternate non-explosive activator(s) became a major challenge for large-scale manufacture of oligonucleotide. The new activator should have all the beneficial characteristic of tetrazole: effective, efficient, less expensive and stable. A new class of high performance, non-explosive activator based on saccharin has been introduced for the synthesis of oligonucleotides[6]. This class of activator fulfills all the beneficial characteristics of tetrazole. Saccharin based activator is efficient and effective at lower equivalent (1:1) compared to tetrazole (3.5:1). The synthesis scheme and structure of this class of activator is given in **Figure 2**.

In contrast to tetrazole, saccharin-N-methyl-imidazolium salt (SMI) has been effective for oligodeoxynucleotide (DNA) and oligoribonucleotide (RNA) syntheses. The use of this reagent has not compromised the quality or impurity profile. This development eliminated the safety and supply issues related to tetrazole use in large-scale oligonucleotide synthesis. The new activator is easily manufactured as a crystalline salt from saccharin and N-methylimidazole in acetontrile.

Minimization of impurities and side reactions

Use of 3-amino-1,2,4-dithiazoline-5-one[7], also known as xanthane hydride solution (XHS), instead of

Beaucage reagent[8] as a sulfur transfer agent, Isobutyric anhydride in place of acetic anhydride for capping, and *tert*-Butylamine[9] for removing cyanoethyl group selectively in order to minimize modification of thymidine, uridine or cytosine nucleosides.

3-Amino-1,2,4-dithiazoline-5-one (XHS)

During the sulfur transfer step, the by-product formed from Beaucage reagent is a reactive compound and this in turn reacts with P(III) triester forming stable P(V) phosphate triester together with P(V) phosphorothioate triester, which forms phosphate diester as contaminant along with desired phosphorothioate diester. The by-product generated from 3-amino-1,2,4-dithiazoline-5-one (XHS) is unreactive; therefore, no side reaction or formation of P(V) phosphate triester takes place. The sulfur transfer reactions using these reagents are illustrated in **Figure 3**.

Use of *tert*-butylamine to minimize acrylonitrile adduct

In the synthesis of oligonucleotides using phosphoramidite, β-cyanoethyl group is used as phosphorus protection. During release of the chain and removal of protecting groups with conc. ammonium hydroxide, cyanoethyl group is released as acrylonitrile. Acrylonitrile has been found to react with N-3 position of thymine or N-4 position of cytosine to form cyanoethyl adduct *via* Michael addition reaction. These adducts are not easily removed by chromatographic purification. However, this problem has been minimized by selectively removing cyanoethyl group without releasing the oligonucleotide chain from the solid support, and also trapping or washing away the acrylonitrile formed. *tert*-Butylamine solution removes cyanoethyl group by β-elimination, and acrylonitrile formed is trapped as *tert*-butylamine reacts with acrylonitrile. Finally, olignucleotide chain is released from solid support and N-protecting groups are removed from the chain with conc. ammonium hydroxide. The **Figure 4** illustrates the reaction and cyanoethyl adduct formation.

Use of isobutyric anhydride instead of acetic anhydride

In the capping step, acetic anhydride is generally used to block un-reacted 5′-hydroxyl group. The exocyclic amino group of guanine (2-position) is generally protected with isobutyryl group. During repeated capping with acetic anhydride, *trans*-amidation with acetic anhydride has been observed. The standard condition for removal of protecting groups is not able to remove the resulting N-acetyl group. This material co-elutes during purification with the desired oligonucleotide as an impurity. Isobutyric anhydride blocks the free 5′-hydroxyl group with no chance for *trans*-amidation side reaction. The use of isobutyric anhydride in capping step indeed prevented formation of this impurity.

The use of 3-amino-1,2,4-dithiazoline-5-one, *t*-butylamine and isobutyric anhydride in various steps of the synthesis process have minimized the formation at least three impurities (phosphate diester, cyanoethyl adduct and N-acetyl-guanosine).

Use of Strong Anion Exchange Chromatography for Purification

The process utilized for purification of oligonucleotides also has impact on cost, throughput, environment (waste generation) and safety. The use of strong anion exchange media for chromatographic purification can be beneficial. The steps involved in this purification described earlier have allowed the scale up of purification from 1 g to ~2 kg. The benefits of strong anion exchange chromatography are outlined below:

Loading Capacity: The loading capacity of strong anion exchange media is significantly higher than reverse phase C-18 or equivalent media and hydrophobic interaction media or weak anion exchange media. Generally, the loading capacity of strong anion exchange media is ~50 mg/mL. This increases the throughput in purification and reduces the amount of chromatography resin required.

Eluting Buffers: Most of the eluting buffers for strong anion exchange chromatography are 100% aqueous salt solutions with either neutral or high *p*H, unlike reverse phase chromatography where buffers contain organic solvents. The use of aqueous buffers minimizes safety issues and hazardous waste generation.

Chromatography apparatus: The purification process using strong anion exchange media does not require high-pressure liquid chromatography instrument. Low-pressure liquid chromatography is sufficient. With reverse phase media, explosion-proof high-pressure liquid chromatography instrument is essential for large-scale purification. The use of low-pressure liquid chromatography reduces the safety concerns.

Ultra filtration and lyophilization

These processes are used for removing the salts and concentration of purified chromatography fractions of

oligonucleotide sequences. UF (ultra filtration) and lyophilization are standard methods of operation in oligonucleotide manufacture, are easily scaleable and do not impose safety or health concerns at larger scales.

Conclusions

Improved process conditions and newer reagents that are suitable for synthesis and purification of therapeutic grade oligonucleotides, up to 4 kg batch sizes have been developed. The advantages of the developed methods have shown that successful large-scale manufacture of oligonucleotide sequences is possible while maintaining high quality, low cost and environmentally safe conditions.

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