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Review

Overview and Recent Advances in the Purification and Isolation of Therapeutic Oligonucleotides

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ABSTRACT: Therapeutic oligonucleotides are a novel chemical modality with unique characteristics that offer significant therapeutic potential. With the latest surge in development, they are paving the way to the market at a rapid pace. To support that, scalable and economically sustainable oligonucleotide production is required. The upstream part exploits the well-studied phosphoramidite chemistry, known from the 1960s, using automated synthesis on a solid support. On the other hand, the downstream processing, which consists of purification and isolation steps, has stayed in the shadow of the more thoroughly studied synthesis, even though it is often regarded as the bottleneck of therapeutic oligonucleotide production. Therefore, this review aims to highlight the most important traditional purification and isolation approaches for the production of therapeutic oligonucleotides that ensure product quality, yields, scale, and cost-efficiency as well as newly emerging separation technologies that could become relevant in the future.

KEYWORDS: *oligonucleotides, purification, preparative chromatography, continuous chromatography, ultrafiltration, freeze-drying*

1. INTRODUCTION

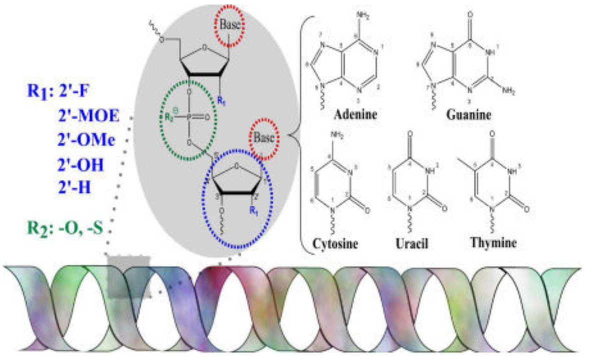

Figure 1. Most common TO structures (circled in blue, sugar moiety; green, phosphate linkage; red, nucleobase) with sugar modifications at 2′ (R$_1$) and potential incorporation of sulfur at phosphodiester linkage (R$_2$) with nucleobases enclosedon the right side of the figure.

Therapeutic oligonucleotides (TOs) are polymeric nucleic acids, short single- or double-stranded ribonucleic acid (RNA) or deoxyribonucleic acid (DNA) molecules with a length of 13−39 nucleotides for currently approved TOs.[1−4] This novel class of therapeutic molecules possesses a unique ability to modulate gene expression via selective Watson−Crick hybridization to the complementary sequence.[1,2,5−7] Their potential to prevent, replace, add, or edit RNA and DNA at the molecular level equips them with the possibility to cure rare and common therapeutic indications including cancer, cardiovascular and metabolic conditions, neurological disorders, and ophthalmic diseases.[1,8−10] In comparison to traditional small molecule drugs, they can also target previously undrugged transcriptome and cellular pathways with much more simplicity as the primary sequence governs specificity and accelerates rational design of these types of drugs.[1,7,11,12] Initial understanding revealed that other factors, not just affinity, might halt the way to successful oligonucleotide drugs, such as chemical stability, resistance to degradation mediated by nucleases, metabolic stability, target site accessibility, delivery, biodistribution, binding to proteins, pharmacokinetics, pharmacodynamics, toxicity, and activation of complement.[13] With the evolution of chemical modifications and drug delivery systems, TOs successfully reached therapeutic potential with better efficacy, safety, and pharmacokinetic profiles (Figure 1).[12,14,15] Replacement of nonbridging oxygen atom in the phosphate backbone with a sulfur atom is called a phosphorothioate modification and was already incorporated into the structure in the 1960s. This simple yet powerful adjustment increases nuclease resistance and also causes higher affinities for the target RNAs and improved pharmacokinetic profile due to better binding to serum proteins. Furthermore, phosphorothioates (PS) contain a stereocenter at each phosphorus resulting in different diastereomeric ratios (R_p or

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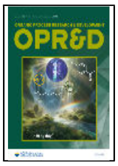

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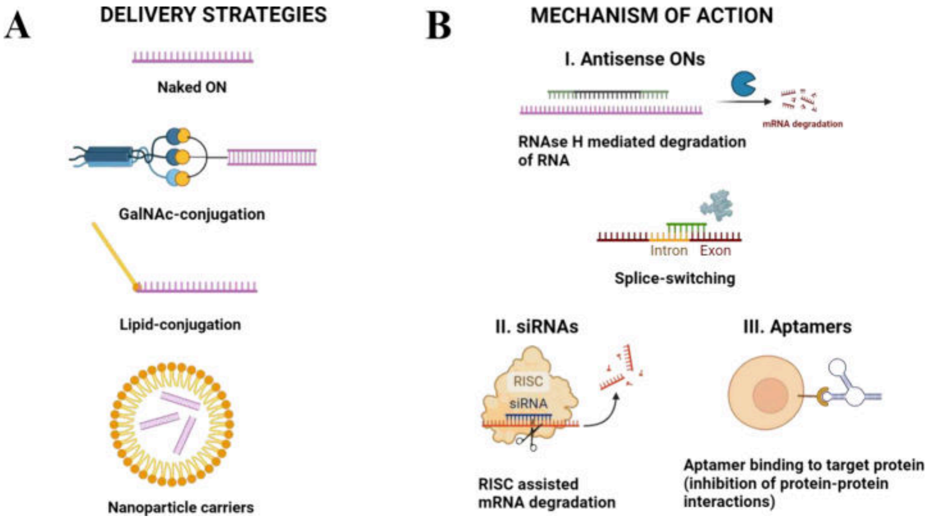

Figure 2. (A) Delivery strategies of TOs and (B) their mechanisms of action.

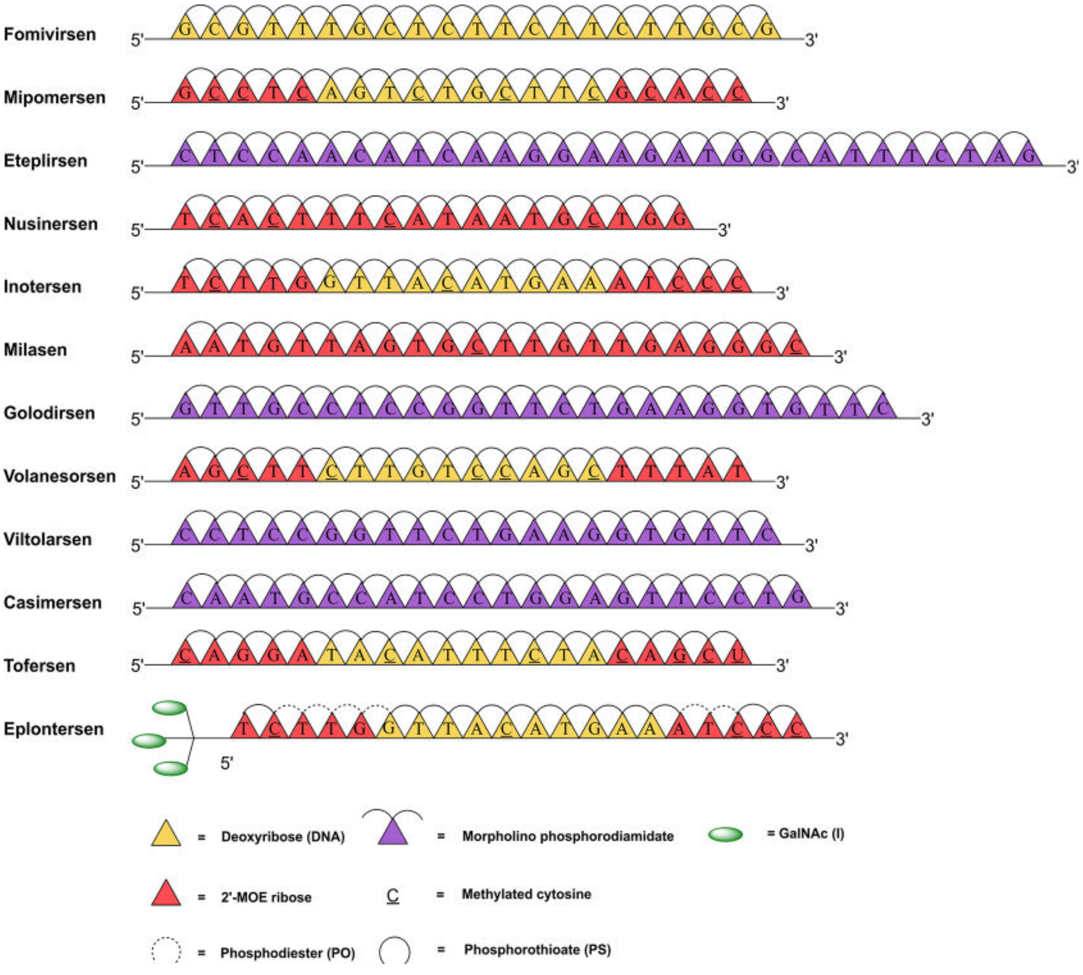

Figure 3. Structures of approved ASO TOs (vaccines are excluded from the scope of this review).

S_p configuration).[1,2,6,16] A phosphorodiamidate morpholino (PMO) backbone (morpholino ring instead of ribose) changes the anionic character to a neutrally charged molecule also providing high nuclease resistance, binding affinity, and positive contribution to cell penetration. Modifications on the sugar moiety mainly focus on the 2′-position with the 2′-OMe, 2′-MOE, and 2-F’ (especially occurring in siRNA), increasing resistance to nucleases and binding affinities.[12,16] Improved binding affinity can be facilitated through base modifications with predominantly methylation of pyrimidine core(5-methylcytosine) or 5-thiazolyl, 5-bromo-, and 5-iodouracil or purine modifications.[12] High molecular weight and a negative charge are intrinsic properties of the synthetic oligonucleotides (ONs), which prevent them from effectively passing through the cell

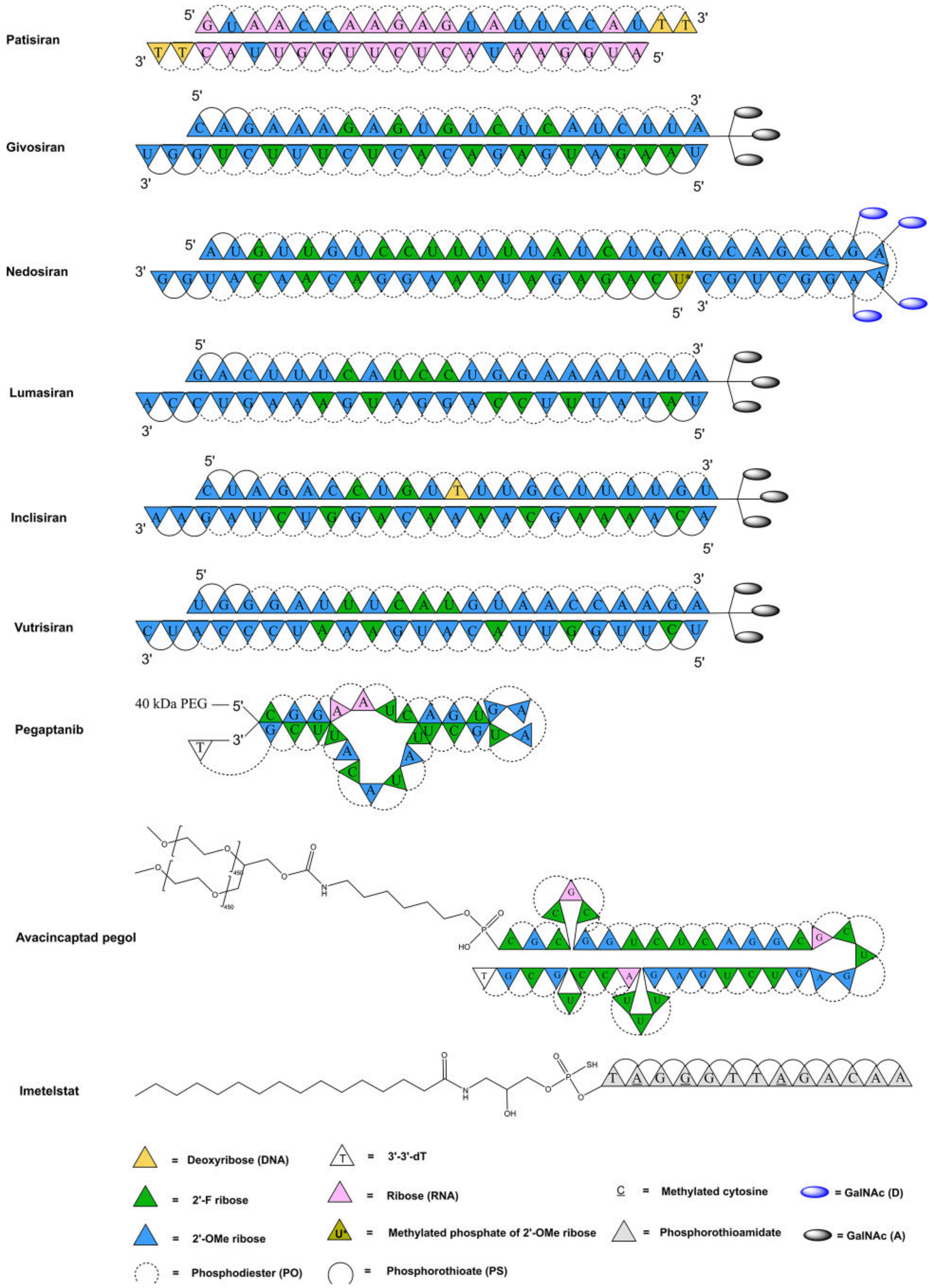

Figure 4. Structures of approved non-ASO TOs: siRNA, aptamers, and imetelstat (vaccines are excluded from the scope of this review).

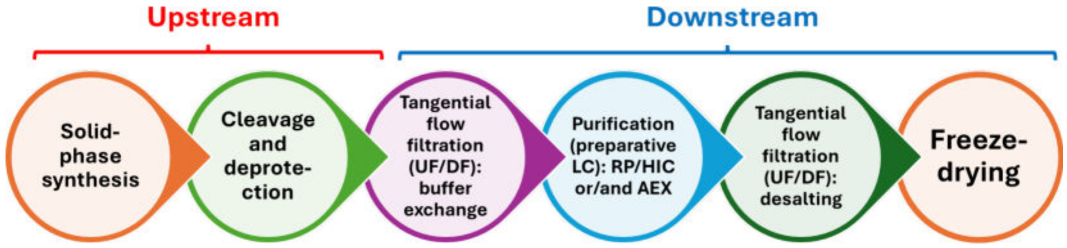

Figure 5. Overview of the TOs processing with upstream and downstream operations.

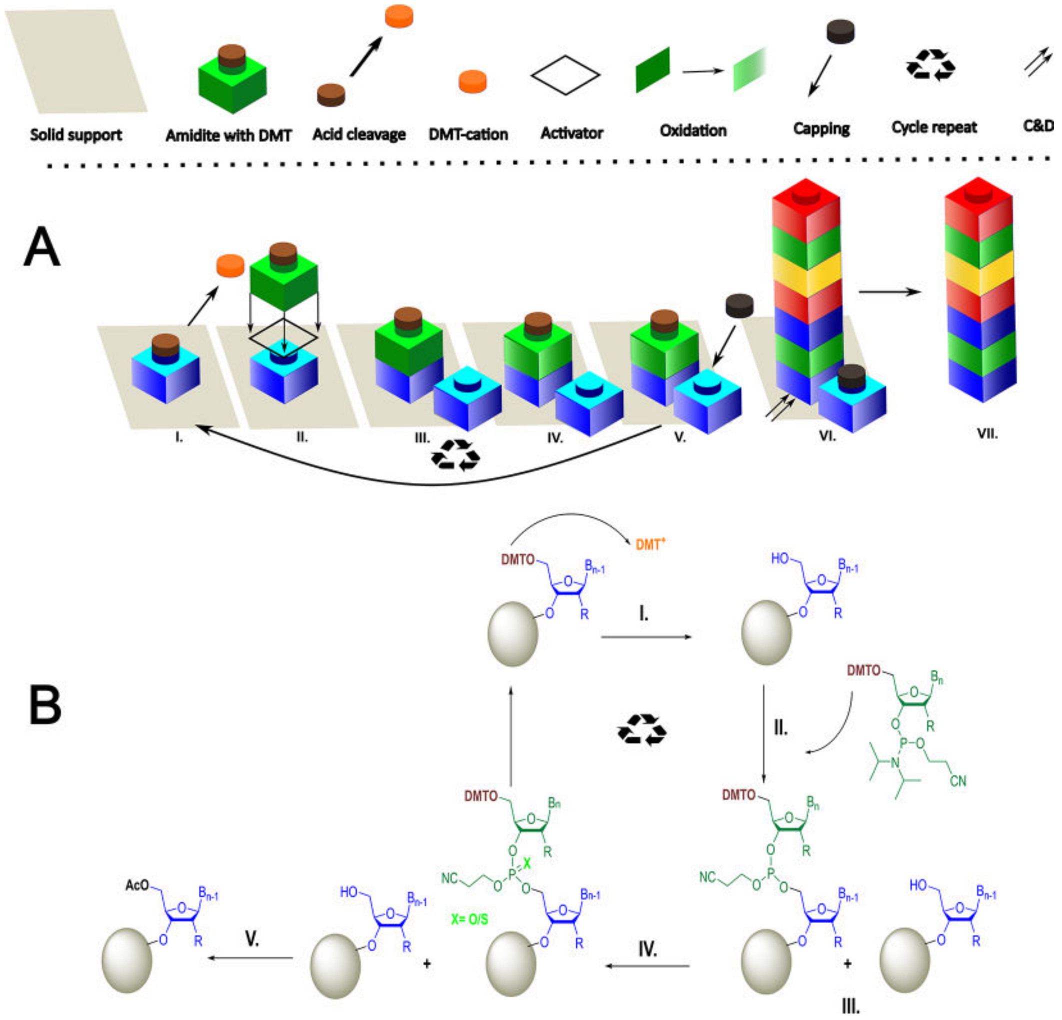

Figure 6. (A) Schematic representation of the ON synthesis with blocks: solid-phase ON synthesis steps (I., detritylation; II., coupling; III., product of coupling; IV., oxidation; V., capping followed by cycle repetitions to elongate the sequence) and cleavage from the solid support and deprotection (C&D; VI.) and final ON (VII.). (B) Solid-phase ON synthetic cycle (steps I.– V.) with chemical formulas.

membranes. Fortunately, delivery strategies such as *N*-acetylgalactosamine (GalNAc)-based conjugation of antisense oligonucleotides (ASOs) and small interfering ribonucleic acids (siRNAs), lipid nanoparticle formation (patisiran), or utilization of cholesterol or other lipids (imetelstat) improve penetration to the target site (Figure 2A).[2,16] Currently approved TOs on the market belong to any of the three classes with distinctive mechanisms of action: ASOs, siRNAs, and aptamers.[14−16] Therapeutic ASOs function through two mechanisms of action (Figure 2B). The first involves degradation of RNA by RNase and is mediated by gapmer–oligonucleotide sequence with a central PS DNA region and 2′-MOE sugar modifications that downregulate the target gene. The second mechanism is called splice-switching, and it enables upregulation of functional gene expression through exon skipping or inclusion.[14] siRNAs enter the cytoplasm and bind the RNA-induced silencing complex (RISC), where the duplex unwinds and the passenger strands degrade. Finally RISC, guided by the complementary antisense strand, induces mRNA degradation (Figure 2B).[17] Aptamers bind to proteins and tend to inhibit protein–protein interactions (Figure 2B).[18] Although the idea of an antisense mechanism of action of ONs was initiated in 1978, it was not until 1989 when the concept started to manifest with some companies increasing attention to the drug discovery.[6] In recent years TOs have positioned themselves as a fast-growing chemical modality with currently 22 TO drugs approved by regulatory agencies (Figures

3 and 4; defibrotide is omitted from the figure due to the mixture of TOs rather than a defined structure, and vaccines are out of the scope of this review) and many more being examined in the clinical trials.[4,13,19−24] In the year 2023 a record of four TOs hit the market with anticipation of a major rise in the manufacturing requirements in the near future.[22,25] To support the growing demand of the TOs field, scalable, efficient, and economically sustainable synthesis and purification processes are required. It has been two decades since purification and isolation technologies of the TOs were thoroughly presented by Sanghvi and Schulte.[26] Recent reviews are scarce and mainly consider the downstream processes together with synthesis (Andrews et al.,[5] Paredes et al.,[16] and Catani et al.[27]) or analytical liquid chromatography (LC) methods with examination mostly in the semipreparative to analytical range also omitting isolation (Zhang et al.[28] and Fornstedt and Enmark[29]), while the review from Studzinska et al. specializes in the ON-based adsorbents for isolation and separation.[30] Although all of these publications provide vital understanding of the TOs manufacturing and analytical characterization, we truly believe there is a clear need for a wholesome and structured review that focuses merely on the downstream processes. This review aims to highlight the most important traditional purification and isolation approaches for the production of TOs along with promising emerging separation methods, providing insights into recent advancements in the field.

2. MANUFACTURING PROCESS OVERVIEW

Production of TOs covers upstream and downstream processing that can be further divided sequentially to

- upstream
  - solid-phase synthesis
  - cleavage and deprotection step
- downstream
  - chromatographic purification
  - isolation

Thus, the process of TO manufacturing consists of solid-supported chemical synthesis followed by cleavage and deprotection (C&D), chromatographic purification, and detritylation (unless performed in the synthesis step), followed by isolation which includes ultrafiltration/diafiltration (UF/DF), concentration, and/or lyophilization (Figure 5).[5]

3. SYNTHESIS OF THERAPEUTIC OLIGONUCLEOTIDES

Oligonucleotide synthesis is based on the well-established principles of automated solid-phase synthesis known for decades, which uses phosphoramidite building blocks (protected nucleosides) for the sequence assembly. During the reaction, reagents flow through the column charged with either controlled pore glass (CPG) already preloaded with the first nucleotide or, an even more efficient alternative, polymeric supports. The nucleotide chain grows from the 3′ to the 5′ direction, and performance of the four-step reaction cycle enables elongation for one monomer. This procedure, which includes solvent washes (acetonitrile) after each reaction step, is repeated until the final protected oligonucleotide chain reaches its desired length (Figure 6). In-line reaction progress detection with UV and conductivity monitoring provides some vital yet limited data to evaluate success of the synthesis.[5,16,27,31] In the first reaction step, solid support is exposed to the acidic wash with 3−10% dichloroacetic acid (DCA) in toluene/dichloromethane, which cleaves the 4,4'-dimethoxytrityl (DMT) protecting group from the 5′-hydroxyl group. Afterward new phosphoramidite is coupled to the freshly released 5′-OH with the use of the activator, which promotes the reaction. Most commonly used activators are 4,5-dicyanoimidazole (DCI), 5-(benzylthio)-1*H*-tetrazole (BTT), and 5-(ethylthio)-1*H*-tetrazole (ETT). Phosphorus atom in its P(III) form is oxidized to a more stable P(V). When PS linkage is required, a sulfurizing reagent is used. Although the coupling step nears 100% efficiency, it never reaches it, leaving some free 5′-OH groups. To prevent nonreacted species to enter another cycle, which would result in $n - 1$ impurities, they are capped with acetic anhydride. The final step—C&D—takes place separately (omitted from the automated synthesizer) and involves release of the oligonucleotide chain from the solid support and deprotection of the remaining protecting groups. Two heavily favored reagents are ammonium hydroxide and methylamine or a combination of both with or without heating. For the *tert*-butyldimethylsilyl (TBDMS) protected riboses at 2′-OH, fluoride ions in organic salts such as TEA·3HF facilitate the deprotection. Speaking with regard to reaction kinetics, cleavage is much faster than the deprotection step. The most base labile residue in the oligonucleotide dictates the use of the C&D reagent. This at first sight seems as a rather straightforward step, but it requires special attention as most impurities induced during C&D are extremely hard to eliminate with subsequent downstream processes. Once the cleavage is done, the support is filtered from the recovered ON solution, washed, and discarded and the filtrate is collected. To confirm the success of the synthesis purity, identity and content are measured.[5,16] Until now solid-phase synthesis has been applied for commercial manufacturing of ONs due to the simple and efficient reaction sequence and high quality of the produced ONs. Cost of the resin, relatively large consumption of expensive chemicals (starting materials and solvents), and more challenging scale-up are limiting factors associated with the solid-phase synthesis. Liquid-phase approaches are not restricted by these challenges and enable unlimited scalability. With evolving technologies such as Ajiphase method (delivers tens of kilograms of product in a single batch), liquid-phase synthesis holds the potential of becoming the method of choice in the future, especially when demand for larger scale arises.[32,33] Assembling long chains of the ON remains challenging for chemical synthesis due to low coupling efficiencies. This obstacle could be circumvented with employing enzymatic ligation steps to combine multiple shorter fragments.[34]

4. IMPURITIES OF THERAPEUTIC OLIGONUCLEOTIDES

After the synthesis, the crude material consists of a full-length product (FLP) accompanied by a plethora of different impurities. The longer the oligonucleotide chain, the larger the number of synthetic steps, which results in an increase in the impurity levels.[35] The iterative nature of the synthetic cycle suggests that even small amounts of impurities in the starting materials will produce a noteworthy amount of impurities in the final product. Impurities originating from starting materials can be divided into three categories: nonreactive (removed with washes during synthesis; least critical), reactive, not critical (built in the ON chain, but removed together with protecting groups without impacting quality of the synthesized product), and reactive, critical (stay in the ON chain, form a different product).[7,16] The platform nature of the TOs facilitates the prediction of common product-[8] and process-related[7] impur-

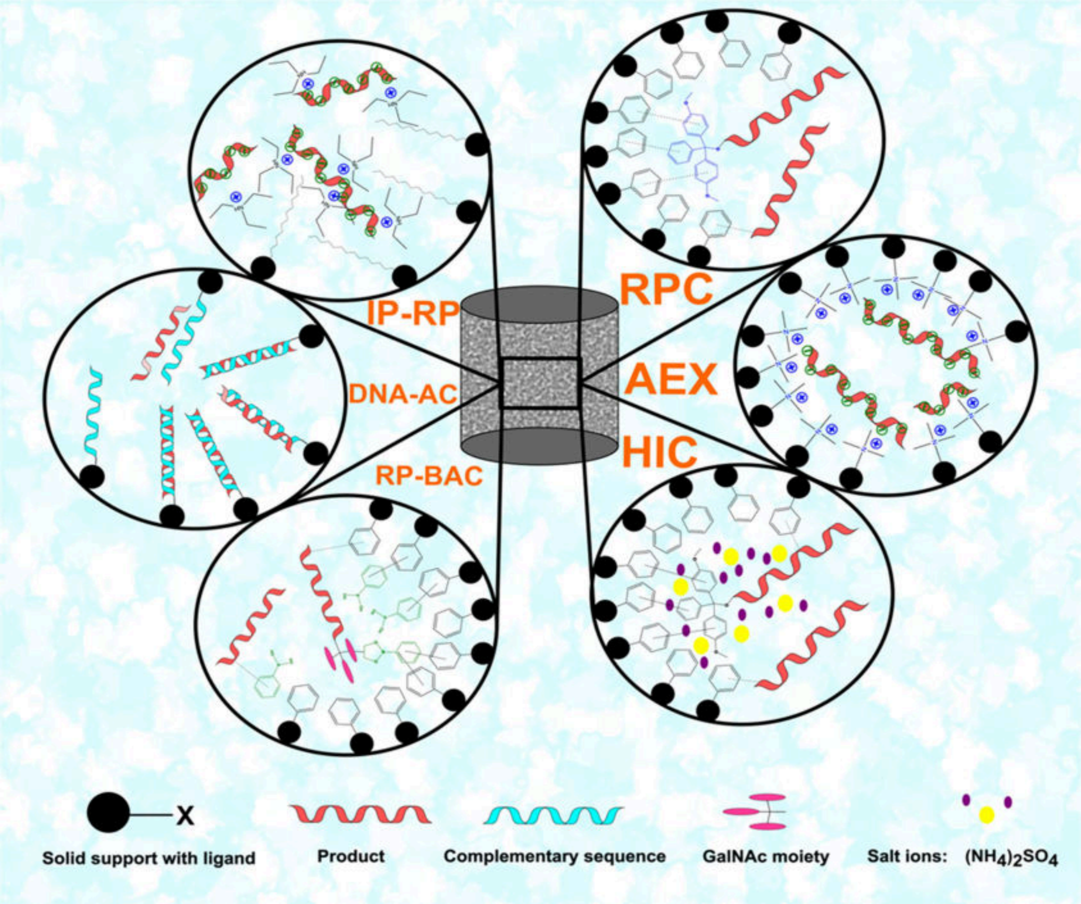

Figure 7. Retention mechanisms of ON to the chromatographic supports of the most widely employed purification techniques (RPC, AEX, HIC; right side) and the ones gaining momentum for the future (IP-RP, DNA-AC, RP-BAC; left side).

ities. Common product-related impurities include shortmers ($n - x$, capped failure sequences, internucleotide cleavage due to degradation), $n - 1$ sequences (group of impurities missing one nucleotide; insufficient detritylation/coupling), longmers (mostly $n + 1$; coupling of two amidites), PS to phosphodiester (PO), trichloroacetaldehyde-modified oligonucleotide (starting material impurity), DMT-C-phosphonate impurity (incomplete oxidation/sulfurization), large molecular-weight impurities (dimers, branchmers, and lambdamers), abasic impurities (e.g., depurination during detritylation), deamination (basic condition in the C&D step), incomplete removal of the protecting groups (DMT, isobutyryl, TBDMS, etc.), acrylonitrile adducts (byproduct of phosphodiester backbone deprotection, reacts with nucleobases), impurities from the solid support, and others.[7,10,35] With the illustration of the upstream processes (oligonucleotide synthesis) and formation of the most common impurities, the importance of the downstream processing steps is evident. Synthesis provides crude material with a limited purity (60−80%, depending on the type and length of the product) containing many impurities with barely distinguishable differences in the chemical characteristics and the relative mass differences in relation to the FLP. Some of the impurities coelute together with the product, making chromatographic purification a challenging task. To hit the required regulatory grade quality of the TOs thorough process optimization will be demanded. In the next section, we will present established methods and growing novelties in the field of downstream processing of TOs.[36,37]

5. DOWNSTREAM PROCESSING OF THERAPEUTIC OLIGONUCLEOTIDES

This section will mostly consider purification and isolation techniques practical for obtaining products of high quality and yield from synthetic crudes with potential industrial use. We will highlight some well-established purification methods such as reverse-phase chromatography and anion-exchange chromatography after years of practical use in industrial settings and emphasize recently reported novel purification methods yet to be further implemented.

5.1. Purification of Therapeutic Oligonucleotides. One may argue that a crude ON sample after synthesis consists of TO and seemingly similar impurities, indicating that a general purification procedure might prove to be satisfactory for the majority of different TO types. The idea behind it, however, seems far-fetched as a distinct purification program warrants essential quality of the TO product. Established platform purification strategies serve as a great starting point in the development, but distinctive product characteristics govern the choice of the applied purification method.[26] Platform knowledge gained from decades of work can be leveraged to guide the process development; however, optimization in a molecule-specific manner to bridge the differences between the elution of the impurities and the product is required.[38] Depending on the scale of the operation ranging from laboratory, to pilot scale, to manufacturing scale and its intended purpose (therapeutic use, structural elucidation, early stage screening, and primer isolation), the purification mode can be selected.[39] In general, we can divide the purification technologies to chromatographic and nonchromatographic ones. Due to larger versatility, greater flexibility, ease of automation, scaling-up and possibility of continuous production, preparative chromatographic separations serve as a golden standard for purification of TOs.[27]

5.1.1. Nonchromatographic Purification of Therapeutic Oligonucleotides. Gel Electrophoresis. Charged nature of the ONs allows them to migrate through polyacrylamide gel in the presence of the applied electric field. Polyacrylamide gel electrophoresis (PAGE) is a traditional method that provides a straightforward approach to purification on the basis of length/size. This approach is more or less adequate only for biochemical applications on analytical and low-preparative scale. Usually not more than 1 mmol is purified with mass recoveries being under 50% due to subsequent extraction from the gel and desalting. However, gel electrophoresis effectively separates full-length oligonucleotide from synthetic failures, where especially length and sequence (C > A > T > G; fastest mobility of C) dictate the mobility through the gel.[16,28,40−42]

SPE (Solid-Phase Extraction). Cartridge purification mimics the principles of reversed-phase chromatography (RPC) as DMT-on ON is loaded to the sorbent in the cartridge. After wash of DMT-off impurities, acid wash to detritylate the sample, and final elution with organic solvent, moderate purity of μmol quantity of product is obtained. This rather primitive small-scale protocols are applicable for fast ON purification with employment of single-use disposable media with selective affinity for the product.[40,43]

Chemical Tagging Approaches. A recent review article by McClain considers innovative approaches for purification with nonchromatographic methods. These protocols focus on designing different chemical tags that promote separation on the basis of altered chemical properties with incorporation of a functional unit at the 5′-end of shortmers during solid-phase synthesis. Described methods include novel chemical approaches such as methacrylamide polymerization, fluoride affinity, biotin−streptavidin conjugation, Staudinger ligation, and inverse electron demand Diels−Alder chemistry.[44] Ren and co-workers proposed a similar approach, where they introduced lipophilic cholesterol−phosphoramidite as a capping reagent as a substitution for acetic anhydride. That drastically elevated the retention times on the reverse-phase silica gel and inversed the separation order compared to the product.[45] Pearson et al. introduced a fluorous dimethoxytrityl group as an affinity tag at the 5′-end to improve retention on the RPC adsorbent.[46] The chemical tagging approaches are still regarded as semichromatographic techniques as chromatography still remains necessary. However, these techniques decrease the dependence on the extensive chromatography, providing simple and efficient solutions. Complex and time-consuming preparation of specific reagents for the synthesis still separates them from practical use.

5.1.2. Chromatographic Purification of Therapeutic Oligonucleotides. Since the 1970s chromatographic methods serve as a powerful tool for purification of ONs.[28] The selection of the chromatographic technique depends on the chemical characteristics of the molecule with respect to its modifications and applications.[27] ONs carry well-defined properties that can be exploited during the purification, such as a negatively charged phosphate backbone, a polar nature due to hydroxyl and amino groups, hydrophobic properties of heterocyclic bases, and the ability to form secondary structures through hydrogen bond formation.[26] Out of many different chromatographic principles, RPC and anion-exchange chromatography lead the way.[5,47,48]

Reverse-Phase Chromatography. Separation mechanism of RPC purification (Figure 7) relies on hydrophobic interaction between the packed bed and the compound, where more polar compounds elute first, but the more nonpolar species are retained on the column and elute with the hydrophobic eluent.[28] To increase retention, the DMT protecting group is intentionally left on the ON (final detritylation is omitted; Figure 6, step I.) and serves as a hydrophobic handle to facilitate retention. This traditional approach renders excellent and robust separation of DMT-off failures that are washed out with moderate levels of organic eluent, whereas the product peak is collected at the higher gradient strength.[5,26] Even when the impurities are as long as the product, the difference between hydrophobic DMT-on ON and DMT-off failures allows powerful separation of the two.[26] Typically employed buffers proposed in protocols from Deshmukh et al.[49] and Sanghvi and Schulte[26] include either 200 mM sodium or ammonium acetate with methanol/acetonitrile as eluting solvent in neutral pH values as elevated pH > 8 diminishes the resolution between the DMT-off impurities and DMT-on product. Increased pH adds to the number of charged sites on the molecule, increasing polarity, which negatively impacts resolution. Buffer choice provides excellent selectivity and capacity with the product in preferred sodium form.[26,49] After purification, the DMT group is removed from 5′-end with acid treatment either with harsher short exposure (5−10 min) in strong acid (1−2% TFA in water) or milder acid (e.g., 10 mM acetic acid, pH 3) for longer time. The goal is to obtain rapid full detritylation, together with minimal depurination levels. Furthermore, on-column detritylation is possible with an additional acid wash step but is rarely performed on RPC columns; however, it is more common in anion-exchange chromatography applications.[49−52] High UV purity samples are obtained with RPC (93−97%) with moderate recoveries and robust performance; however, decreased loading compared to anion-exchange chromatography, potential pressure limitations in scale-up, and large consumption of organic solvents are limitations to be taken into account.[5,26,49] Stationary phases with polymeric design (polystyrene divinylbenzene, cross-linked polydivinylbenzene, and polymethacrylate) use C4, C18, or phenyl ligands to assist the separation and show superior stability, loading, and recovery compared to the silica-composed stationary phases.[5,30]

Anion-Exchange Chromatography. One of the most widely used methods for separation of biomolecules, especially proteins and different nucleic acids, is anion-exchange chromatography (AEX).[16,26] This method leverages the anionic nature of the multiple phosphates on the backbone of ON to build strong attraction forces with the positively charged amine ligands on the solid support (Figure 7).[26,28] In theory, the higher number of charges results in increased retention compared to that of lower charged species. In the context of ONs, shorter deletions that bear reduced overall charge up to $n - 1$ impurities are well-separated, as are also PO impurities. Improved separation of $n - 1$ failure sequences from FLP occurs in fully PO ON compared to fully PS molecule due to reduced hydrophobicity of PO in contrast to PS.[26] In recent work from Kobl et al., they try to illustrate the importance of understanding the ion-exchange mechanism and explore the optimization strategies with modeling and simulation.[25] In essence, AEX is fundamentally different from typical adsorption principles. When one anion on the solid support is replaced by the other of the different kind, the solution properties, such as pH change (if the hydroxide ions are involved), influence the number of charges on the molecule and affect the elution behavior. Furthermore, there is no guarantee that all charged sites on the target molecule will actually gain access to bind to the resin via an ion-exchange mechanism. The authors proposed a step-by-step methodology to investigate the model parameters with maximum output of the data with a minimum number of performed experiments. Even if the process is modified, information related to determination of the charge vs pH curve of the molecule, resin, and resin−buffer interactions can be reused. This and many more challenges are answered in the mentioned publication.[25] Loaded ON in DMT-on mode exhibits strong interaction with the AEX stationary phase, requiring high salt concentrations for its elution. To avoid that, the product is washed with strong acid (e.g., 0.4% TFA in water), to eliminate the protecting group, neutralized with base (e.g., 10 mM NaOH) and later eluted with linear salt gradient. The advantage of the more common AEX purification, which involves sample loading, low conductivity salt wash, gradient salt elution, and column regeneration, is to avoid deprotection, which may induce the formation of depurinated impurities and cumbersome product isolation. Various applications employ AEX as an orthogonal method to complement RPC in a two-step (1, RPC; 2, AEX) purification, especially when a high-purity product (e.g., TOs) is demanded.[25,26,49] Recently Nosengo et al. established an integrated batch process for the purification of a single-stranded DNA oligonucleotide. This technology combines two chromatographic methods by automatic transfer of the selected fraction of the peak from the first column to the second column. Compared to two separate single-column purification runs, this method was proved to be superior in the reduced analysis times and complete automation of the process. The developed process involved a sequence of RPC and AEX chromatographic methods (RPC-AEX or AEX-RPC), with both showing improved yield compared to the corresponding batch. The order of RPC-AEX is practically beneficial as no inline dilution is required.[53] This strategy is implemented mostly for antisense TOs; however, siRNAs are generally purified only with AEX without the protecting DMT group. Usually, aqueous buffers in the alkaline part of the pH spectrum (sodium phosphate/NaOH) with eluting salts (NaCl/NaBr) are employed, and addition of an organic modifier might also influence the elution regime. To avoid formation of advanced structures (e.g., multimers, aggregates) of self-complementary or GC-rich ONs, combination of basic pH, elevated temperatures, and organic solvents maintains at least partially if not fully denaturing conditions.[16,26,28] High pH eluents might yield additional charge to accomplish discrimination of the product to the impurities, but, in certain ONs containing high pH-labile 2′-OMe and 2′-F nucleosides, that should be avoided.[28] Finding the right balance between denaturation for purification and pH/heat-generated on-column degradation must be defined to have optimum method performance.[16] In their publication Goyon et al. elucidate why sodium bromide provides narrower peaks compared to other possible elution salts (NaCl, ammonium chloride) when purifying phosphorothioates.[54] In the phosphorothioate group, charge is mostly placed on the sulfur, which is regarded as a soft base. The electrostatic affection between the sulfur and positively charged ligands on the solid support can be most readily terminated with addition of another soft anion, in this case the bromide anion.[54] AEX adsorbents can be classified into two groups, where strong anion-exchangers (SAX) retain their positive charge independent of the pH (quaternary amines); on the other hand, weak anion-exchangers (WAX) use secondary or tertiary amines, where surface ionization changes in accordance with pH.[30] Two SAX polymeric resins with the same ligand chemistry (quaternary amine) but different support characteristics polystyrene divinylbenzene (Source 15/30Q) or poly(methyl methacrylate) (TSK Gel SuperQ-5PW) are well-established as industry standards and offer high surface area due to small particle size and substantial porosity. Other examples of SAX resins are Q Sepharose HP/FF, ToyoPearl GigaCap, Workbeads 40Q.[16,29] That results in high loading capacity, and great resolution affords high final sample ON purities. Both types show excellent stability throughout a wide pH range, therefore it is possible to perform denaturing purification and even directly load the ammonium-based crude sample right after C&D.[47,48,55,56] The solid supports with quaternary amine ligands provide complete separation of PS ASO impurities, PS products, and their unmodified analogues. On the other hand, strong retention on the resin increases processing time. The most widely used ligand for WAX is diethylaminoethyl (DEAE) resin, and in this case, pH plays a pivotal role in the ON retention on the adsorbent. It reaches the highest surface charge at pH 4; however, at pH 9 it drops to only 50% of the maximum value. This restricts establishing a slightly alkaline environment, where formation of the ON secondary structures on the column is prevented. The ability of the DEAE stationary phase to interact mainly with 5′ and 3′ ends, where interaction with interiorly oriented bases is small, proves useful for separation of $n - 1$ failures and positional isomers. Increasing the pH of the mobile phase allows separation based on the chain length (at 8.5−9.5) or ON sequences with different positions of one base (at pH = 10.5).[30] There are various advantages that make AEX purification superior to RPC. The aqueous nature of the eluent system usually avoids organic solvents, the equipment and the solid supports are less expensive, time-consuming detritylation is omitted, loadings are increased compared to RPC, and the method also separates PO impurities from ON. Finally, the fraction pool is less concentrated and additional desalting is required, which are the two limitations inferior to RPC.[26] When producing double-stranded ONs, each crude strand is processed separately; however, Noll and co-workers reported a protocol, where they successfully purified a preannealed siRNA duplex in the nondenaturing way rather than each single strand alone.[48] Furthermore, they observed that not only anion exchange but also a secondary mechanism influences the elution of the single strands in comparison to the duplex. Besides electronic interactions, van der Waals and hydrophobic interactions between the nucleobases on the ON and solid support also add to retention forces. Major differences between single strands and the duplex exist with regard to binding to the solid support. Single-stranded ONs are much more flexible compared to the duplex, with their hydrophobic surfaces (nucleobases) readily available for binding. On the other hand, the rigid nature of the double-stranded ON, where nucleobases are stacked to opposite base pairs, shows limited potential for formation of secondary hydrophobic interactions. That is exactly why single strands elute before the duplex on the polystyrene divinylbenzene solid support; however, on the polymethacrylate solid support, the elution order is reversed—indicating that hydrophobic interactions allow stronger retention of the single strand in the second case.[48]

Monolithic Chromatography. One of the major limitations in preparative purification of larger molecules is slow mass transfer and low binding capacity. The solution to circumvent these bottlenecks could be to exploit monolithic stationary phases.[26,55] This type of support consists of one continuously structured block with interconnected pores, in contrast to particle-based supports. Inside the monolithic structure larger through-pores exist, which enable convective flow of feed molecules; however, smaller mesopores within the monolithic backbone provide a high surface area.[26] It is to be noted that monoliths are hard to manufacture and, in terms of popularity, do not follow the success of particle supports. Their main advantages are excellent flow-through properties, which render high operational throughput and significantly decrease processing times. However, due to the low surface area compared to particulate matrixes, it is almost impossible to establish desired resolution to separate closely eluting ON failures from the FLP. One example where the technology proved to be superior compared to particulate supports was presented in the work of Kazarian et al.[56] With AEX monolithic stationary phase, they successfully purified a guanine quadruplex that adsorbed too strongly on the polymeric AEX beads resulting in low recovery.[56] CIM monolith columns might possess the ability to resolve ONs with minor changes in the length.[57] Other examples of this technology are also well-documented in the literature.[58−60]

Displacement Chromatography. More than 20 years ago the pioneering work from Cramer's group rendered another tool to tackle ON impurities.[61−63] AEX displacement method employs a displacing molecule that possesses higher binding affinities to the resin than any other in the crude mixture. After the column loading, the molecule displacer pushes other bound components downstream, with those possessing higher retentions acting as displacers to the ones with lower retention. Finally, after all product species elute, the column is regenerated with high salt concentration eluent.[26,64] Different low-molecular-mass displacers (e.g., amaranth and saccharin) for AEX purification were successfully identified and implemented.[61−63] The main advantages of this technology are excellent product purity and a high final concentration. Furthermore, the product elutes at low salt concentration; therefore, desalting could be omitted.[26,63] As Andrews and others emphasized in their work, this advantageous approach might again be exploited in the future, especially to decrease solvent consumption.[5] Lajmi et al. managed to develop a membrane chromatography AEX procedure in the self-displacement mode as a polishing step for purification of therapeutically useful ASO. It proved to be a robust, scalable, and high-throughput method, offering fast, high-resolution separation, avoiding any column packing or packing validation.[65] This promising work has yet to convey its potential to be firmly positioned as an alternative to more established purification protocols.

Hydrophobic Interaction Chromatography. Similarly to RPC purification, hydrophobic interaction chromatography (HIC) exploits the DMT group for its binding to the solid support, but the mechanism focuses on the "salting-out" effect to expose hydrophobic surfaces of the DMT group (Figure 7). HIC shows its superiority to the RPC in two aspects: ammonia solution can be directly loaded to the column without preprocessing and it only requires aqueous-based solvents. The solvent system includes a strong chaotropic agent, usually ammonium sulfate, which enforces a hydrophobic interaction between DMT and ligands on the support. Gradual decrease to low salt concentrations elutes the product as almost only an aqueous ON solution and is perfectly convenient for potential direct AEX loading in the next step. If desired, ON can as well be detritylated on the column with acid treatment as described before.[5,26,49] In the recent patent literature, Gronke and others show potential reduction of $n - 1$, PO, and CNET impurities in the final product with HIC purification.[66]

Ion-Pair Reverse-Phase Chromatography. RPC purification entails the presence of a DMT protecting group to establish retention to the hydrophobic support; however, ion-pair reverse-phase (IP-RP) chromatography follows the ion-pair mechanism, which promotes direct loading of the detritylated ON.[49] Compared to RPC or AEX, IP-RP exploits two separation mechanisms at once: charge−charge interactions as well as hydrophobic interaction, ultimately behaving as a mixed-mode separation type (Figure 7).[29] The ion-pair reagent (IPR) (alkylamine or arylamine) in the mobile phase adsorbs to the hydrophobic stationary phase. It bears a cationic character induced by adjusting the pH; therefore, it forms electrostatic interactions with multiple negatively charged groups on the ON.[29,30] Furthermore, increased selectivity can be achieved with formation of non-electrostatic interactions between ON and the stationary phase. IP-RP chromatography can provide excellent resolution for certain impurities, particularly those with minor structural differences or closely related species, due to its combination of charge-based and hydrophobic interactions; furthermore, it can be coupled with MS detection. Length of the IPR, its branching properties, and interaction with the stationary phase govern the retention mechanism. The higher the number of charges, the higher the retention of the target molecule, indicating length-based separation; however, the influence of hydrophobic interactions with nucleobases makes it sequence-dependent as well. Although massively employed for the utilization of analytical tasks, preparative IP-RP has not yet taken a step forward.[29] Few scientific publications have focused on preparative IP-RP techniques. Fortunately, recent comprehensive work encompasses theoretical and practical studies for better fundamental understanding of analytical as well as semipreparative IP-RP separations targeting selective elimination of impurities.[29,67−70] In these publications the understanding gained from analytical IP-RP development to establish optimal semipreparative separations focused on the choice of IPR and the solid support. Tributylammonium acetate (TBuAA) is the preferred ion-pair selection to increase separation of impurities as it suppresses selectivity for diastereomers.[29] Enmark investigated factors influencing separation of diastereomers, where trimethylammonium acetate (TEtAA) proved to obtain the highest selectivity for diastereomers.[68] Adequate ion-pair concentration should render at least the same number of cations to compensate for the cumulative number of negative charges on the ON. Stationary phase with phenyl chemistry behaved better than C18 (widely applied for analytical separations), C8, and C4 columns. Comparative study with mass-overloaded injections of fully PS 16-mer on C18 and phenyl columns showed successful purification due to quantifiable displacement of the early eluting shortmers with both ligand types. Narrow elution profiles were achieved in both cases with a high 16-mer purity and yield. The phenyl column better separated late-eluting impurities such as a 17-mer, indicating slightly superior performance to C18 column.[67] Two modes of gradient decrease the electrostatic potential to elute the product decreasing ion-par concentration at constant cosolvent (organic solvent) or decreasing cosolvent concentration at constant ion-pair level. Besides TBuAA, other explored IPR involves triethylammonium acetate (TEtAA), dibutylammonium acetate (DBuAA), diisopropylammonium acetate (DIPAA), TMeAA, ammonium acetate (AmA), tripropylammonium acetate (TPrAA), diethylammonium acetate (DEtAA), and hexylammonium acetate (HAA). With a plethora of options, in terms of IPR chemistry, one can choose the optimum IPR for the sequence- and structure-dependent separation of ONs. Elongation of the alkyl chains of tertiary amine by one methylene group at the time results in a linear increase of the logarithm of the retention factor. Decreasing the carbon chain length increases diastereomer selectivity. In these cases, TEtAA (TMeAA evaporates quickly) is the IPR of choice. Provided that increased resolution of the shortmers from the product is required, using larger amines (e.g., TBuAA) proves superior to shorter ones, especially for PS ONs that cause broadening of the parent peak.[29] After the purification, IPR, which is harmful due to toxicity, must be eliminated from the solution requiring an additional isolation step (e.g., ultrafiltration) to obtain the preferred sodium form of the product unless another chromatographic step still follows.[29] Another limitation is the potential formation of *N*-nitrosamines from IPR.[71] Fillon and co-workers assessed purge factors for ON-related process-related impurities but have not taken into account IP-RP purification as a purification technique that was studied.[9] IP-RP chromatography stands out as a powerful preparative method, but has yet to be fully established in the large manufacturing processes, where AEX still prevails.[29]

DNA Affinity Chromatography. In theory, principles of the affinity chromatography (AC) are based on the specific interactions between two biological agents, where one of them is immobilized on the stationary phase as an affinity ligand that possesses the ability to retain complementary structures (Figure 7).[72] There is a scarce amount of data available in the field of affinity chromatography related specifically to ONs; however, the first immobilization of DNA to silica support dates back to 1990.[73] DNA affinity chromatography (DNA-AC) leans on the selective base-pairing mechanism between analyzed ON and the sequence on the chromatographic support. Recent pioneering work from Studzińska et al. opens the door for potential future approaches with studies of retention mechanisms and preparation of different stationary phases.[73] In one of their recent studies, they successfully covalently attached a DNA sequence to a silica-based aminopropyl support. Different ON sequences with varying degrees of complementarity were tested with three chromatographic modes. Out of the tested retention mechanisms, HILIC (hydrophilic interaction chromatography) and AEX did not show adequate selectivity between the ONs; however, affinity chromatography with a sodium chloride buffer system and applied temperature gradient showed excellent separation power. Salt ions decrease the negative repulsion forces between the charged backbones, increasing the retention. The rise in temperature majorly influences the selectivity due to destabilization of the formed duplex between the immobilized strand and loaded complementary product with breaking of the hydrogen bonds.[73] This invaluable approach has yet to be explored in nonlinear preparative screening to further prove its value. Potential challenges lie in the synthesis of a tailored stationary phase and its stability. These two would drive the cost of separation significantly emphasizing potential utilization in manufacturing.[30] Nevertheless, the potential ability of the method to decrease the levels of impurities that mainly elute under the main peak in the currently established approaches (RPC, AEX) could be extremely beneficial. Especially some critical impurities with minimal differences in mass (classified in groups III and IV according to Capaldi, e.g., base modifications that provide different complementarity) might be decreased with alternative AC purification.[10]

Boronate Affinity Chromatography. The most prevalent regime for the conjugation of TOs that enables the selected delivery to the liver relies on the GalNAc strategy. Currently, six GalNAc-conjugated TOs are on the market and many more in the clinical trials.[74] Besides the conjugated product, some unconjugated impurities are formed and must be eliminated. Despite the fact that GalNAc possesses some hydrophobic properties, affinity to the RPC solid support is lower than in the case of DMT, resulting in lower resolution between the conjugated and unconjugated peaks. Furthermore, GalNAc lacks any functional group bearing charge that could be exploited with AEX. Lately, Gabriel and colleagues have found an elegant strategy, where they leveraged the principles of boronate affinity chromatography to establish reverse-phase boronate affinity chromatography (RP-BAC).[75] Its mechanism relies on establishing a reversible covalent bond between boronic acid and cis-diol containing species (Figure 7).[76] In alkaline conditions, or better said, when the pH is higher than pK_a of the employed boronic acid, the latter transforms to the tetragonal (sp^3) boronate anion, which can then form five- or six-membered rings with cis-diols. If the pH is lowered back to acidic values, where pH of the solution is lower than pK_a of the acid, the interaction is broken and the acid exists in trigonal (sp^2) orientation.[77] In the work of Gabriel et al., the authors managed to improve affinity of the GalNAc conjugate to the RPC resin with addition of different aryl/alkyl boronic acids that formed complexes with cis-diols on the GalNAc moiety subsequently increasing the hydrophobicity.[75] Mobile-phase pH was set at alkaline values higher than 8 to endow the binding. With the use of boronic acids as mobile-phase additives, they significantly improved the resolution between the conjugated product and unconjugated impurities.[75]

Other Chromatographic Methods. Although the above-described preparative chromatographic methods prevail, some interesting LC approaches that are largely implemented only for analytical purposes should be discussed as well. First of them—HILIC (hydrophilic interaction chromatography)—is a chromatographic method that separates molecules in accordance with their polarity. Phase partitioning, electrostatic interaction, and hydrogen bonding are mechanisms influencing separation using the HILIC method.[78] First implementation of the HILIC method for ON separation dates back to the 1990s, and it has recently been recognized as an alternative characterization method to the IP-RP.[27,79] Head-to-head comparison of the two reveals increased selectivity of IP-RP to HILIC; however, with progressing ON modifications at polar residues, an additional complementary method could prove to be beneficial.[78] When coupled to MS, IP-RP shows lower sensitivity as IPR suppresses the signal.[27] Goyon et al.[78] and Lardeux et al.[74] also explored HILIC for diastereomeric separation. To date, no HILIC purification has been reported; however, with further investigation of the retention mechanism, it might prove more relevant in the future.

Recently Hayasida et al. investigated applicability of supercritical fluid chromatography (SFC) for separation of tetramers with different PS contents.[80] With improved diffusion properties and lower viscosity due to carbon dioxide in the mobile phase, high separation throughput can be obtained. In their work, the authors showed that careful selection of additives results in good peak shapes and resolutions and will shift their focus to prove that longer, more hydrophilic ONs still hold promise.[80] Its

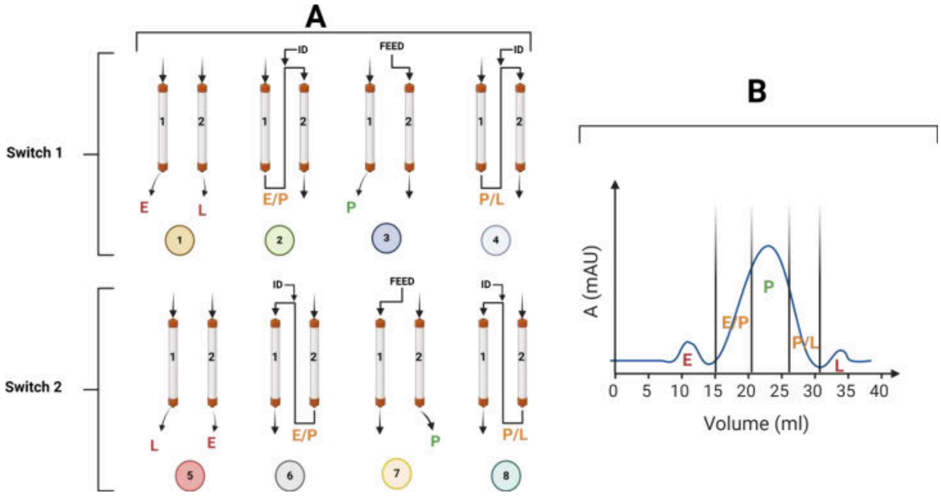

Figure 8. (A) Schematic representation of MCSGP switch with four major operating steps. (B) Example of preparative chromatogram where MCSGP has potential to improve process efficiency (P, product; E, early eluting impurities; L, late-eluting impurities; ID, in-line dilution).

distinctive retention mechanism, high-throughput, eco-friendliness, and cost effectiveness could potentially be exploited for the preparative approaches in the future.[80,81]

Rare examples of size exclusion chromatography (SEC) reported in the literature indicate its minor relevance. Due to limited resolving power it cannot render the high quality of the TO products that is often required and in the best scenarios serves as a complementary method.[27] Its nondenaturing property is leveraged for analysis of double-stranded ON to characterize the formation of impurities during the annealing of siRNAs.[79]

Mixed-mode chromatography (MMC) leverages anion-exchange and reverse-phase mechanisms in one separation, avoiding multistep purification with careful tuning of elution conditions (altering buffer pH, buffer concentration, and organic modifier).[82,83] Anion-exchange mechanism behaves as the predominant one to the reverse phase; therefore, AEX supports with a certain degree of hydrophobicity can be employed for mixed-mode separations as illustrated in the work from Kazarian and co-workers.[83] Supposing the benefits of MMC further excel in affording purifications of high-quality material, they might surpass single-mode purifications

5.1.3. Multicolumn Chromatography (MCC). Until now our center of attention was focused on single-column applications known also as batch chromatography, which is the predominant processing setup for industrial approaches. It is noteworthy to mention once again that many chemically similar impurities significantly coelute with the product, which demands careful fractionation of the elution peak. In order to achieve required regulatory grade quality of the product (usually purity well above 90%), side fraction cutting gives back diminished returns with 20−50% yield losses. One way to tackle the diminishing return is to manually recycle the overlapping sections at either early- or late-eluting ends, which include mostly the desired product, but where the impurity levels are still too high for final pool selection. This rechromatography step comes at a great cost, is labor intensive and time-consuming due to decreased productivity, increased buffer consumption, and intermediate sample storage capacity, and requires additional quality steps; therefore, the side fractions are usually discarded at the commercial stage. The inverse relationship between the purity and yield, also called the purity to yield trade-off, exacerbates purification even more in the field of the ONs, where separation between the FLP and the impurities is demanding to achieve in the first place and the expensive upstream synthesis adds to that as well.[27,36] One way to circumvent this challenge is implementation of multicolumn or continuous chromatography, where a series of identical columns are connected through a system of switching valves that imitate the motion of the stationary phase in the opposite direction of the eluent flow. This continuously operated technique produces significant process improvements.[27]

Simulated Moving Bed Chromatography (SMBC). One type of MCC is the simulated moving bed chromatography (SMBC), where feed and eluent inlets, product, and impurity outlets move downstream of the multiple column train with the valves operating in predetermined switching intervals that govern the continuous processing cycles. Major limitations of the SMBC are that it can only be run in the isocratic mode and separate two molecules.[26] Almost two decades ago, Schulte et al. exploited SMBC for the separation of DMT-on ON from DMT-off failures.[84] The main challenge was to successfully separate the species with the isocratic elution; therefore, even though they obtained good resolution, a high content of DMT-on product eluted in the DMT-off fraction.

Multicolumn Countercurrent Solvent Gradient Purification (MCSGP). Single-column operation in the batch mode prevents achieving high purity and yield at the same time due to overlapping regions of the product and the impurities.[85,86] Specific method optimization approaches such as decreasing the particle size of the resin, gradient slope, or the amount of the loaded crude material, and increasing column length, improve the selectivity of the operation but for the cost of diminished productivity, longer run times, and higher solvent consumption.[86,87] Another more encouraging approach to circumventing challenges related to single-column techniques is multicolumn countercurrent solvent gradient purification (MCSGP). This technology appeared in 2006 with the pioneering work of Morbidelli and Aumann, and it has paved the way for several other applications showing the potential to revolutionize the area of purification of biomolecules.[85,87] The main advantage that separates the MCSGP operation from standard preparative batch processing is the automatic internal recycling of the partially purified side fractions, which enables achieving high

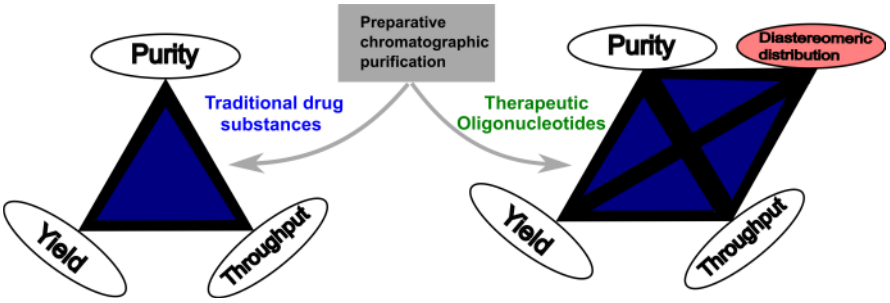

Figure 9. Chromatographic triangle for traditional drug substances and "chromatographic rhombus" with diastereomeric distribution as a 4th cornerstone in the case of chiral TOs.

purity and high yield at the same time.[85] During the operation, the idea is to collect only the central selection of the peak (P) that meets the specification for purity (Figure 8B). On the other hand, the overlapping regions of early- (E/P) and late-eluting (P/L) impurities and the product (Figure 8B) are recycled to the second column.[86] This positional switching of the inlet/outlet column valves simulates the movement of the stationary phase. The side fractions are adsorbed and go through another linear gradient method to restart the separation. Initially, MCSGP setup included 3−6 columns; however, the reduction to only two identical columns greatly increased the flexibility of the operation and minimized its complexity.[36,87] MCSGP operation is a cyclic process, where columns are either interconnected (two steps of recycling overlapping regions) or operated in batch mode. Importantly, the composition stream leaving the first column is diluted in-line with the compensation buffer. This reduction in the concentration of the modifier in the recycling stream to its value at the start of the gradient facilitates adsorption on the second column. Loading of the column differs from batch operation. It is not performed in a single step but in three separate steps with uptakes of E/P and L/P with fresh feed (crude sample after synthesis) loading in-between.[86] MCSGP can be divided into the initial phase and the four operating states 1−4 (Figure 8A, top):[36,37]

0. Loading of column 1 with feed during a method startup before the switch or from the previous switch in MCSGP. Column 2 is being regenerated to remove late-eluting impurities (L) or is already clean at the start of MCSGP.
1. The first batch phase, elution of the early eluting impurities (E), is taking place on column 1, and these are directed to waste. Column 2 is being cleaned and re-equilibrated.
2. The first interconnected phase, the overlapping region E/P from column 1, is diverted to column 2 and diluted in-line to restore the binding condition.
3. The second batch phase, the product fraction, is being eluted from column 1; column 2 is loaded with fresh feed. The amount of feed loaded to the second column corresponds to the exact amount of product that leaves the first column.
4. The second interconnected phase, overlapping region P/L from column 1, is diverted and loaded to column 2 and diluted in-line to restore binding condition.

After completion of the above-described steps, which present the first switch, the second switch starts (Figure 8A, bottom). All of the steps from the first switch are repeated in the second one with the only difference being in the interchanged column positions. One cycle of MCSGP includes two switches and operates continuously until steady-state is reached (purity and recovery of the collected pools are almost the same) and all feed material is purified.[36,85] Designing an MCSGP with proper operating conditions is much more demanding than for batch chromatography. Careful selection of product collection window and recycling intervals is integral to optimizing the product yield and preventing the accumulation of impurities inside the column.[37] To define the operating parameters of the MCSGP, single column purification is performed beforehand. Fractional analysis of the product peak provides vital information regarding the distribution of key impurities and the product to correctly set the MCSGP method. When transferring a batch method to MCSGP, operating parameters such as loading and gradient slope keep their values; however, switching times are balanced to carefully correspond to phase borders of the eluting species.[85] However, this time-based settings approach could be exchanged with more efficient and robust UV-based dynamic control that has just recently been proposed by Fioretti et al.[88] With the implementation of the AutoPeak function, recycling and collection windows in MCSGP can be adjusted as a response to the system perturbations. Study showed that under variations in processing parameters (changes in loading duration, feedstock purity, and operating temperature) without AutoPeak control, yields were decreased to 20% compared to those where AutoPeak was applied. This technology manages to maintain critical quality attributes in the specification automatically, ensuring product quality and decreasing the potential risk of product loss during the operation. This significant breakthrough has possibility to be implemented to real-time process control of any MCSGP operation.[88] Advantages such as decreased buffer consumption, better utilization of the resin, automation, increased throughput, abolishing the need for offline side fraction recycling, and a lower burden on quality control put MCSGP in the prime position for even more widespread use. Compared to the single column operation, the complexity of the system increases drastically with MCSGP as well as the requirement for its validation and maintenance. Weldon et al. investigated establishment of MCSGP for RPC purification of GalNAc-cluster ON. Head-to-head comparison of MCSGP to batch chromatography indicated 73.6% increase in relative yield at 94.2 area % purity.[36]

5.1.4. Diastereomeric Composition during Purification.

The primary goal of successful purification is to obtain a high product purity with high yields as quickly as possible. Balancing purity, yield, and throughput is challenging, and meticulous attention must be paid to find the optimum separation condition. However, acknowledging the fact that PS and morpholino TOs exist as a complex mixture of diastereomers[89] with discrete properties, expanding the "chromatographic triangle" into "chromatographic rhombus" seems reasonable (Figure 9).[90]

Typical fully phosphorothioated 20-mer contains more than half a million diastereomers.[29] Differences in the chemical properties between S_p-isomers and R_p-isomers are being reported, with S_p-isomers possessing higher hydrophobicity and ion character compared to the R_p-isomer analogues.[91] It has been known for years that diastereomers display different biological activity; therefore monitoring the diastereomeric profile during manufacturing is of utmost importance to prevent variations in efficacy, as even small process changes might alter the composition.[74,92] Furthermore, based on the interactions with regulatory agencies, Roussis et al. noted in their work the need to demonstrate stereochemical reproducibility in the manufacturing process.[93] A good example that sheds light on the topic is mongersen, a 21-mer antisense ON with all PS modifications, which was produced for multiple years of clinical development. All batches characterized with common analytical methods seemed to be identical, until ^{31}P NMR spectroscopy revealed differences that were in accordance with differences in biological activity.[92] LC characterization methods showed changes in elution profile between different diastereomeric mixtures, which is to be expected as they possess different chemical properties influencing retention.[91] During a common purification process, the best separation of impurities from the FLP is desired; however, together with discriminating the chemical differences between these two groups of compounds, separation of the stereoisomers is also obtained. Since both reproducible purity and diastereomeric distribution are required, even minor changes in the selection of fractions for pooling are able to affect it.[74] With narrower peak-cutting some part of the diastereomeric composition will be lost; on the other hand, wider fraction selection strategies will diminish the purity. This calls for the development of a robust purification process, where conditions are fine-tuned for an optimum outcome in the purity as well as the reproducible distribution of diastereomers. Suppression of diastereomer separation prevents the loss of stereoisomers but significantly diminishes the separation of the impurities.[74]

Large-Scale Manufacturing. Large-scale TO manufacturing has yet to fully develop as only one of the currently approved TOs (inclisiran) did not receive orphan drug status since it treats a more common disease, which requires much larger commercial quantities than all of the other approved TOs. Even without previous manufacturing experience in the TOs, prior knowledge somehow still enables to set up large-scale GMP production with little laboratory-scale process development. Multiproduct facilities justify high capital investment of the manufacturer to utilize the full capacity of the plant; however, some companies still rather turn to contract manufacturing organizations to implement the scale-up for them. Up to date, out of all of the purification methods described above, only RPC, AEX, and HIC are employed for commercial applications. The selection of the method relies on the product characteristics and the capability of the method for eliminating impurities, as well as the limitations of the existing processing line. Equipment failure is the number one cause of the deviations; therefore, process simplification might minimize those errors. Fundamentally, the platform nature of the TOs ensures seamless transition to manufacturing scale providing consistent yields and time-effective production.[94]

5.1.5. Model-Based Optimization of Purification Methods.

Previous chapters highlighted the complexity of the ON separation from the molecularly similar impurities, showcasing principles of various methods to effectively purify the product. The general objective of the purification performance relies on maximizing the yield for a given purity, which is greatly affected by the operating conditions (e.g., crude loading, flow rate, wash duration, gradient slope, pH, temperature, column dimensions, resin type, etc.). Defining critical process parameters comes as an integral task for optimum performance; however, an in-depth investigation of the main input−output relations comes at a cost of enormous experimental burden provided all possible combinations are to be tested. Fortunately, the recent surge in the predictive mechanistic simulations of the preparative separation systems allows investigation of extensive conditions without testing them all experimentally.[25,95] Not many literature reports focus on preparative purification of the ONs, but the number of empirical and mechanistic studies is populating.[29] From the theoretical point of view, modeling of preparative chromatography is much more demanding compared to that of analytical chromatography. Due to the limited surface capacity of the stationary phase, operation of the column is done under overloaded condition, meaning that further increasing the sample load decreases the adsorbance of the product. Competition among various types of components for the same binding sites leads to band interactions and band contaminations. Normally analytical chromatography is described using linear or nonlinear equations, but modeling of overloaded nonlinear chromatography demands solving more advanced partial differential equations describing the mass balance related to convection, dispersion, and paramount knowledge about the components adsorption isotherms.[69] Multiple recently published papers strongly emphasize the importance of mathematical modeling for both analytical and overloaded preparative methods, particularly in the context of IP-RP purification. Leśko et al. presented a retention mechanism and a mathematical model that predicted overloaded concentration profiles in IP-RP using sodium benzenesulfonate as a model molecule.[96] Later this work was followed by building and testing the models that predicted overloaded concentration profiles for different slopes of the organic modifier and column load on three crude 16-mer ONs with varying degrees of phosphorothioation as well as $n - 1$ and PO impurities.[97] Furthermore, a unified retention model was developed by Enmark et al. applicable for IPR gradient as well as cosolvent gradient mode. Comparison of the two showed that IPR gradient provides higher yield and productivity together with decreased cosolvent consumption.[69] Bagge et al. determined adsorption isotherms on the 60 and 300 Å pore size C4 reverse-phase columns for analytical and preparative modes. With the use of a geometric model, the estimated accessible surface area was determined on the different columns. Final results indicated that preparative columns with a 60 Å pore size achieved a 10% increase in productivity compared to those with 300 Å packing material, despite the 60 Å columns having a surface area five times larger.[98] A comprehensive model of ion-exchange ON purification was developed by Kobl et al. and tested on three case studies from leading pharmaceutical companies (Section 5.1.2, Anion-Exchange Chromatography).[25] Most recently, Taguado Menza et al. established a model-based approach for optimizing performance of the RP single-column chromatographic purification of a 20-mer DNA sequence. After validation of the equilibrium-dispersive model in only five experimental runs, the impact of the collection window, loading, and gradient duration on the yield and productivity of the process at purity threshold set at 99.0% were

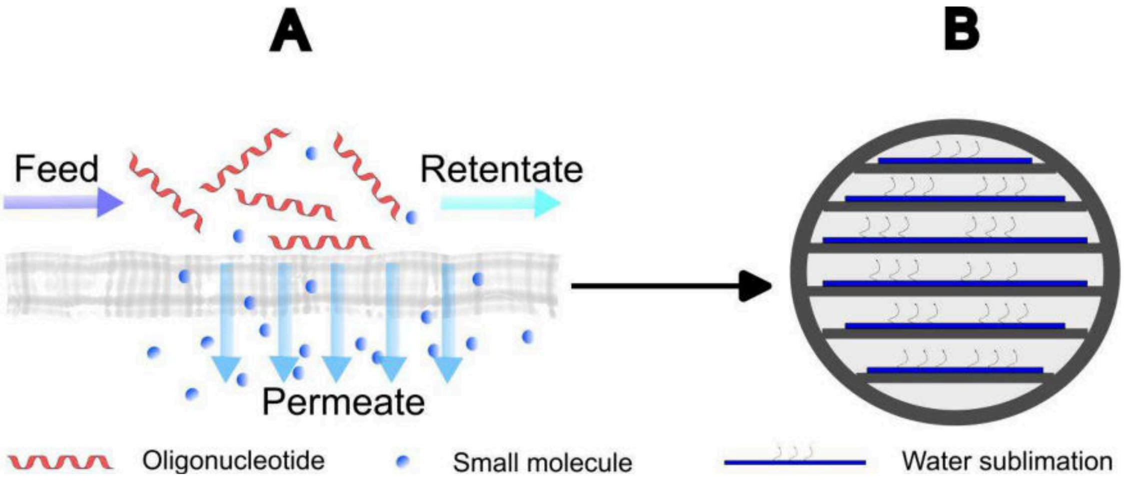

Figure 10. Isolation steps involving (A) TFF and (B) freeze-drying.

investigated. In addition, the developed model strategy showed excellent operation when applied in the MCSGP operation, further improving yield and productivity.[95] Vetter-Joss et al. conducted a mechanistic modeling design to develop and optimize a single-column method, transferred it to MCSGP, and finally scaled it up to commercial production.[99] Although the presented methods purified synthetic peptides rather than the ONs of our interest, the study suggests potential future implementation of these modeling approaches from laboratory method development to production scale, including in the domain of ONs. Mechanistic modeling proves to be of paramount importance for rapid process development, process understanding, helping with troubleshooting, and allowing the implementation of more complex innovative multicolumn technologies.[25]

5.2. Isolation of Therapeutic Oligonucleotides.

Although the order of the narrative might suggest otherwise, isolation steps do not solely belong to the activities after purification but are also performed, if necessary, on the crude sample (after synthesis and C&D) and in-between the chromatographic processing. Methods vary according to the given molecule, scale of operation, and the approach chosen by the manufacturer. Techniques such as ultrafiltration/diafiltration (UF/DF), precipitation, thin-film evaporation (TFE), and lyophilization render a wide range of possibilities for concentrating, desalting, buffer exchange, elimination of smaller impurities, and desolvation.[9,16,100,101] After chromatography the fraction pool of eluted product exists as a dilute, aqueous solution that undergoes desalting and buffer exchange either with more traditional precipitation or tangential flow filtration (TFF).[5] Using alcohols such as ethanol or 2-propanol with the salt promoters (sodium salts, e.g., sodium acetate) or with the help of cooling, ONs precipitate out of the solution. To maximize yield and prevent coprecipitation of salt, optimization of conditions for each ON type and concentration would be required. However, these straightforward approaches work well for a research-oriented environment with no significant equipment demanded or with the aim to convert the product to final solid.[5,16,100] It was not until the past decade that TFF, which was previously seen more as a toolbox for biologics, finally surpassed other techniques in ONs isolation.

5.2.1. Ultrafiltration/Diafiltration.

Many of the recently approved TOs contain at least one UF/DF operation, marking general acceptance of the filtration technology by the regulatory authorities. With its predictable operability, relatively well-known process development and scalability, accessible equipment, and materials, it has established itself as an industry standard.[100] UF/DF applies the TFF technique to its operation. It uses a set of pumps and tubings to circulate a sample (feed) to a porous membrane with the determined molecular weight cutoff, where small molecules such as water, solvents, inorganic and organic molecules, and byproducts pass through (permeate), whereas larger species (ON) divert back to the feed vessel as part of the retentate (Figure 10). As the process advances, the volume decreases, concurrently eliminating small molecular impurities and concentrating the product (UF). Provided the volume stays constant throughout the operation as water or buffer is added to either desalt or exchange ions, it is DF.[5,16,100] A rule of thumb suggests to opt for at least two times smaller molecular weight cutoff (MWCO) than the size of the filtered ON to prevent product loss. Membranes are composed of polymeric materials such as PES (poly(ether sulfone)), regenerated cellulose (RC), polyvinylidene fluoride (PVDF), and nylon. In general, literature in the field of the UF/DF of ONs is scarce; therefore, it is even more satisfying to see the pioneering work from Gronke and co-workers that managed to explore different processing parameters under various conditions and provide some valuable conclusions.[100] They managed to show that the model ASO (7.2 kDa) with moderately high concentration (40 mg/mL) shows a linear relationship between transmembrane pressure (TMP) and permeate flux. This evaluation points to low filtration resistance and a low potential for membrane fouling. Interestingly, permeate flux and therefore sieving coefficients (the ratio between the concentration of the product in the permeate and the concentration of the product in the feed) were larger, when implementing 1 kDa PES in contrast to 3 kDa RC membrane, meaning that material type and its microstructure/channel design might play a pivotal role rather than just MWCO. Depending on the process requirements, there is a trade-off between the process efficiency and product retention, for which one might have to decide for. Sieving coefficients when employing a 3 kDa membrane stayed low for different ASOs (5.7−7.2 kDa) with varying chemistries. At increased concentrations, sieving factors decrease, which might be due to the higher electrostatic repulsion between the ASOs in the membrane layer. Gronke et al. estimated gel concentration (c_g) of the ON—the maximal concentration, where precipitation would occur on the membrane surface—to 200 mg/mL.[100] At this theoretically determined point the flux would be zero. Concentration of the product increases the resistance of the filtration, which decreases the permeate flux. Optimal operating concentration range of the 16−20-mer ON lies between 20 and 50 mg/mL. In this range, the flux should be maintained at constant levels with excellent yields. Viscosity is another parameter that plays a role and might increase a pressure drop and lower the flux and recovery. The majority of ASOs stayed under 15 cP viscosity at concentrations up to 200 mg/mL, but the ON with six-carbon linker terminating in an amine moiety at the 5′ end experienced much higher viscosity showing sequence and chemistry dependence. Not just ON concentration but also conductivity significantly influences the permeate flux. Only water might be used for the desalting operation, but the data suggest that providing at least a minimum conductivity level (>5 mS/cm at 60 mg/mL) is advised for maintaining adequate flux to prevent its decline. UF/DF of typical ASO carried out on 3 kDa RC membrane showed great performance with a TMP range of 25−40 psi, with seven DF volumes required for diafiltration to obtain exceptional 95% recoveries. Generally, ONs do not tend to adsorb on the membrane, therefore ultrafiltration membrane cassettes can be recovered and reused again; however, 3 kDa membranes might retain endotoxins (approximately 5−10 kDa in size) potentially limiting reusability or adding a need for stricter cleaning and sanitization protocols. In addition, with the buffer exchange during diafiltration, product can be formulated to the preferred matrix for the drug product processing or for the stable storage of the drug substance (DS). It was shown that monovalent ions enter the membrane pores; however, divalent ions tend to remain in the retentate either owing to strong binding to negatively charged ON, limited sieving due to repulsion forces with the charged membrane, or because of Gibbs−Donnan effect. In many instances, UF/DF is the last step before the final lyophilization. The obtained final active pharmaceutical ingredient (API) as a rule exists in the sodium salt form; therefore, introduction of sodium during the DF is necessary to exchange other cations potentially occupying phosphate linkages. Provided the ON exists as a sodium salt, performing DF with water would still preserve its nature, however, it would severely limit the flux levels as seen priorly above. On the other hand, maintaining the salt level post-UF/DF is required with sodium content as a final DS specification.[100] An elegant solution was again raised by Gronke et al. to increase the permeate flux and achieve a high concentration of the ON in the retentate post UF/DF, avoiding additional concentration before the lyophilization to achieve an adequate cake structure. It is based on the introduction of additives to increase conductivity while maintaining higher permeate flux levels. Ammonium acetate consists of ammonium and acetate species, which are both volatile in neutral states and can be eliminated during lyophilization. Furthermore, ammonium acetate has its pH range close to the neutral values (6.9−7.7), which are desired for the ON drug product (DP).[102] Furthermore, UF/DF enables successful clearance of process-related impurities that might still remain in the feed before the operation.[9,100] This versatile technology, however, has its limitations. One of them is large water/solvent consumption; the second one relates to maximum concentration that can be achieved before the gel layer starts to significantly hinder the permeate flux. Especially subcutaneous DS demands higher initial concentrations that might not be feasible for the UF/DF to target. Thin film evaporation (TFE) has potential as an alternative operation after UF/DF with its ability to remove water with continuous evaporation, where the sample flows through cylindrical walls, and relatively short processing times. Complementary rotary evaporation might potentially degrade the product due to higher temperatures and has limited scalability as well as vacuum distillation.[101] Following isolation, there might be additional operations necessary to obtain the final structure of the desired ON. With regard to the double-stranded products, such as siRNA, separately prepared single strands are annealed. Combining solutions of the strands in the equimolar ratios, mixing, slow heating to denaturing temperatures, and moderate cooling spontaneously hybridize the strands to duplex form.[5] Besides annealing postsynthetic conjugations aim to circumvent delivery to tissues and pharmacokinetic challenges. Possible covalent attachment includes ligands such as cholesterol, vitamins, carbohydrates, peptides, and proteins.[103] Most common conjugation ligands of the marketed drugs are PEG (polyethylene glycol), which enable prolonged half-life and increased retention in the eye (pegaptanib, avacincaptad pegol) and the GalNAc moiety (well-spread across the field; conjugated either to siRNA or ASO). However, it should be noticed that some ligands are bound beforehand to the solid support and not postsynthetically.

5.2.2. Lyophilization. In general, the last step in downstream processing is the isolation of powder API that exists as a low-density, hygroscopic, electrostatic, amorphous solid (Figure 10).[5,16] First, the ON solution placed on the trays is frozen, and sublimation is induced with applied vacuum and gradually delivered heat. When the temperatures of the shelf and the product are equal, primary drying is usually finished. Throughout this step, most of the water sublimates; however, some amount remains adsorbed and is eliminated with stronger vacuum and further heating (secondary drying).[16] Already intensive downstream operations are even more lengthy as the lyophilization might take up to 1 week to finish. This energy-intensive and cumbersome procedure comes as a significant bottleneck to the manufacturing, therefore considerations of its actual need come as no surprise.[5,100] Alternatively, spray-drying could be a viable option, but would render significantly lower yields.[5] Marketed TOs are in liquid form either in vial or as a prefilled syringe. Knowing that the final manufacturing operation on the API part is sublimation of water and the first step of the DP compounding is dissolution in water, one would naturally question the need for lyophilization. Advantages and disadvantages are well-explored by Muslehiddinoglu et al.[101] Powdered API shows superior chemical and microbial stability compared to the solution API; however, ON also exhibits relatively good long-term stability in solution (or even better when frozen). When the API in the solution is frozen, stability during transition phases (freezing/thawing) must be assessed. In general, there is a low risk of product degradation associated with freeze/thaw cycles, but it still remains product-dependent. However, challenges might arise with the scale-up of the operation due to localized formulation changes within cryovessels or pH shifts of certain excipients (e.g., phosphate buffers) as a result of cryo-concentration and successive precipitation of the buffers. The simplest way for the DP manufacturing involves producing ready-to-fill API solution during final UF/DF. This procedure skips initial dissolution and compounding steps that are generally required during DP manufacturing for the powdered API. Leachables could be of concern when stored in solution; however, as mentioned above, to produce solid API, one has to go through more complex manufacturing and time and energy consuming lyophilization.[101] As a response to the publication from Muslehiddinoglu[101] et al., Wetter et al.[104] enlighten regulatory challenges that come with the TO as API in solution.[104]

6. SUMMARY AND FUTURE OUTLOOK

This review comprehensively examines the well-established and recent novel downstream processing techniques in the field of ONs. With the TO area believed to grow even more in the future, not just in the production requirements (potentially further expanding from orphan drug status to treating common chronic diseases, e.g., inclisiran) but also in the chemical diversity (modifications on integral parts of the molecules, conjugations, etc.); only gradual implementation of the novel purification and isolation strategies warrants future success. Often regarded as a bottleneck of the TO production due to its complexity and duration, downstream processing currently enables providing a high-quality API with moderate yields to satisfy the requirements. Most widely employed purification methods (RPC, AEX, and HIC) have a proven track record and ability to eliminate most shortmers, longmers (up to single-mer differences), and other structurally distinctive impurities on the large-scale manufacturing level. By integrating various chromatographic techniques (such as IP-RP, which does not require DMT protection and shows excellent separation power, RP-BAC, which selectively leverages the affinity between the GalNAc and boronic acids, and DNA AC, which shows sequence selectivity, to name a few of them mentioned in this review), we might successfully address the challenge of eliminating the coeluting impurities that usually remain under the main peak. Using experience from the platform-gained knowledge on the purification program will warrant economic feasibility, but larger specific changes in the TO structure might accelerate the implementation of different approaches. Improved product purity would improve the probability of success of the drug candidates in the clinical trials, preventing off-target toxicity. Furthermore, eliminating at least some impurity groups eluting under the main peak would simplify the usually complex and tiresome analytical characterization. Future research of the purification methods, mainly LC, should prioritize expanding the scope from analytical or semipreparative levels to preparative scales. As seen in this review, many significant achievements have been achieved in recent years but are yet to be further studied to show potential for future utilization. Novel engineering approaches, such as MCSGP, are knocking on the door to complement current batch chromatography setup and might very well prove superior, when the demand for increased batch sizes arises. Complex processing equipment might pose a risk, knowing that equipment failure is the number one cause for deviation in the process, but significant improvements in the yield and throughput might be too good to turn down. Isolation relies on the UF/DF which established itself with predictable operability and relatively well-known process development, expressing versatility with desalting, buffer-exchange, and concentration options. When a final API in solid form is required, freeze-drying is the method of choice. To avoid this time-consuming and energy intensive operation, storing API in a frozen solution or preparing ready-to-fill solution API within the UF/DF step serves as feasible and convenient alternatives. Improvements in the computational resources simplified the building of mechanistic models, where computer simulations enable process understanding without extensive experimental work. Future implementation of machine learning and artificial intelligence might even further foster method development in the years to come. The present review underlines major characteristics of ONs and enlightens the entire production process with a thorough review of purification and isolation methods. Although a more traditional processing platform remains the current standard, novel techniques, and optimized methods may confer improved process efficiencies in the near future.

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Author Contributions

The manuscript was written through contributions of all authors. All authors discussed the results and contributed to the finalization of the paper. All authors have given approval to the final version of the manuscript. CRediT: A.A., conceptualization, writing—original draft, and visualization; Z.Č., conceptualization, project administration, supervision, and writing—review and editing.

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