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Current trends and innovative applications in the synthesis, characterization and purification of oligonucleotides

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Abstract: Oligonucleotides (ONs) are gaining increasing importance as a promising novel class of biopharmaceuticals. Thanks to their fundamental role in gene regulation, they can be used to develop custom-made drugs (also called N-to-1) able to act on the gene expression at pre-translational level. With recent approvals of ON-based therapeutics by FDA, a growing demand for high-quality chemically-modified ONs is emerging and their market is expected to impressively prosper in the very next future. To satisfy this growing market demand, a scalable and economically sustainable ON production is needed.

In this paper, the state of the art of the whole ON production process is illustrated with the aim of highlighting the most promising routes towards the auspicated market-size production. In particular, the most recent advancements in both the upstream stage, mainly based on solid-phase synthesis and recombinant technology, and the downstream one, focusing on chromatographic techniques, are reviewed. Since ON production is projected to expand to the large scale, automatized multi-column technologies will reasonably be required soon to replace the current ones based on batch single-column operations. Cutting edge purification solutions, based on continuous chromatography, will be thus presented in the last part of this review.

Keywords: Oligonucleotides, biopharmaceuticals, drugs, gene therapy, solid-phase, chromatography, MCSGP, continuous chromatography

1. Introduction

Nucleic acids play a fundamental role in all living organisms, being the main actors in duplication and protein formation processes. These events occur thousands of times every day in the human organism, with hundreds of errors committed by the cells at different levels. Fortunately, cells have efficient control systems, able to protect the organism from these errors (*i.e.* mutation) and from their effects [1]. However, even these control systems sometimes undergo failure. When this happens, a degenerative process may arise, with the consequent appearance of a disease [2]. Such pathologies are manifold and often severe such as cancer, muscular dystrophy and cystic fibrosis, as well as cardiovascular and neurological diseases.

Different pharmaceuticals have been developed over the years to deal with these pathologies. These are mainly divided in two classes: small molecules and macromolecular drugs [3]. The former are often characterized by poor selectivity and specificity, which macromolecular drugs can significantly improve, thus paving the way to high therapeutic indexes with reduced dosages and adverse effects [4]. Among this class, biologics such as peptides and antibodies (e.g., monoclonal, transgenic, recombinant, polyclonal) have tremendously grown in interest in the last few years, as indicated by the steep increase in the number of products that entered clinical trials [5]. However, these therapeutics have some limitations especially related to their poor pharmacokinetics, with only the 20% of the dose eventually interacting with the target [6]. Moreover, these compounds act on the cellular process after the protein translation and this may be a limitation especially for the treatment of degenerative processes [7]. On the other hand, the possibility of developing drugs which would influence the cellular processes at an earlier point in time could bring significant advantages [8]. For example, silencing a gene instead of continuously treating the proteins it is responsible for, could provide a deeper and more effective treatment of the degenerative processes. This new perspective, based on gene therapies, is made possible by the maturation in the genome sequencing techniques [9] [10] [11]. Genome sequencing is indeed useful not only for the diagnosis of rare diseases, but also for the development of customized therapeutics, within the so-called N-of-1 approach [12] [13].

These new frontiers in custom-made macromolecular drugs able to act at a pre-translational level provide the main motivation for the increasing interest and efforts in the development of oligonucleotides (ONs) as possible therapeutic agents [14]. Oligonucleotides are short nucleic acid chains, which are made unique by their peculiar potential in the regulation of gene expression. Indeed, an increasing number of synthetic ONs is being developed for a wide range of applications and medical indications, with a few of them in the final stages of human clinical trials and Food and Drug Administration (FDA) approval.

To date, nine ON-based therapeutics, listed in **Table 1**, have been approved by FDA [15] [16] [17] [18] [19], and more than 180 ON-based clinical trials are reported on Clinical.Trials.gov, 37 of which are indicated as Active/Recruiting.

Table 1 – FDA approvals of ON-based therapeutics per year, as of 20th December 2019 [15] [16] [17] [18] [19].

Name (Brand Name)	Agent	Clinical Application	FDA Approval
Fomivirsen (Vitravene)	Antisense 21nt oligo	CMV Retinitis	1998
Pegaptanib (Macugen)	RNA aptamer 27nt oligo	Age-related macular degeneration	2004
Mipomersen (Kynamro)	Antisense Gapmer 20nt oligo	Familial hypercholesterolemia	2013
Eteplirsen (Exondys 51)	30nt oligo, splice modulating	Duchenne muscular dystrophy	2016
Defibrotide (Defitelio)	Mix of oligos, 9-80nt	Hepatic veno-occlusive disease	2016
Nusinersen (Spinraza)	Antisense 18nt oligo, splice modulating	Spinal muscular atrophy	2016
Patisiran (Onpattro)	siRNA	polyneuropathy	2018
Inotersen (Tegsedi)	Antisense 20nt oligo	polyneuropathy	2018
Milasen	Antisense oligo	Batten disease	2019

These achievements, and particularly the encouraging results obtained by antisense oligonucleotides (A-ONs) in clinics, have triggered an increasing demand for high-quality ON-based therapeutics.

In most cases, ON-based drug candidates include AONs, small interfering RNA (siRNA), antagomirs, microRNA (miRNA), decoys, aptamers and CpG ONs. They differ in their chemistry, chemical structure and mechanism of action so that a few classifications have been suggested. A frequently used one is based on their target: AONs, siRNA, antagomirs and miRNA target RNA, while decoys, aptamers and CpG oligonucleotides act on proteins.

Generally, ONs are macromolecules whose synthesis and purification are characterized by several difficulties. Primarily, unmodified ONs may undergo degradation by nucleases when introduced into biological systems and show unfavourable cellular uptake and biodistribution [20] [21]. Therefore, chemical modifications, e.g. the phosphorothioate backbone and sugar modifications, are often required to provide nuclease stability [22]. Over the years, many of these issues have been addressed by developing suitable reaction pathways and methods for the preparative scale ON synthesis have been developed. This enabled to reduce significantly the production costs compared to the first pioneering attempts. Significant advances in synthetic technologies, mainly based on the development of optimized reagents, have allowed improved yields and purities of the ON-based produced drugs. For example, the replacement of the natural phosphate internucleotide with the phosphorothioate linkage (often referred to as first generation backbone modification) and several sugar modifications (referred to as second generation oligonucleotide modifications) have provided enhanced pharmacokinetic and pharmacodynamic properties [23] [24]. However, effective delivery of ONs to their intracellular sites of action remains a major challenge [25] [26]. Numerous drugs containing modified nucleotides are currently under development, but not all of them are suitable for clinical applications. This mainly depends on the potency and toxicity of ONs on one side, and on the availability of large-scale synthetic protocols on the other. Among all modified nucleotides, to the best of our knowledge, only seven chemistries are involved in the ONs currently employed in clinical or preclinical tests [27]. These are schematically summarized in **Figure 1**. The first antisense drug (Fomivirsen) approved by FDA was a 21-base phosphorothioate DNA (PS DNA). Today, the majority of therapeutic ONs incorporate the PS backbone modification to improve biological stability and cellular uptake [28] [29]. PS is usually used in combination with second-generation sugar modifications. The most common sugar-modified therapeutic ONs are 2'-*O*-RNAs, such as 2'-*O*-methyl (OMe) or 2'-*O*-methoxyethyl (MOE) RNA. Compared with normal DNA or RNA, they improve binding affinity and stability toward endonuclease digestion [30] [31]. Four MOE drugs have been FDA approved: Mipomersen and Inotersen are 20-mer PS-2'-MOE gapmer, while Nusinersen and Milasen are uniformly modified PS-2'-MOE 18- and 22-mer respectively. 2'-*O*Me and 2'-F-RNA are two other sugar modifications included in the therapeutic FDA-

approved aptamer Macugen: all the purine ribose sugars are 2'-OMe and the pyrimidine ribose sugars all 2'-fluorinated. The substitution of 2' oxygen with fluoride increases the binding affinity of 2'-F-RNA more than the 2'-OMe or 2'-MOE [32]. Another phosphate modification in clinical trials is the N3′-P5′ thiophosphoramidate (NPS) linkage in which the 3' bridging oxygen is replaced with an NH group offering good affinity for the RNA target and improved nuclease resistance [33]. Imetelstat a 13-mer based on NPS chemistry is currently in phase II/III for myelofibrosis and myelodysplastic syndromes [34]. Spiegelmers (L-RNA) are a new class of oligonucleotide therapeutics built from non-natural L-nucleotides: the mirror-image configuration confers enhanced plasma stability and immunological passivity. From the list of Spiegelmers, three are currently in several active clinical trials [35]. Several locked nucleic acid (LNA) are employed in various stages of clinical or preclinical studies [36]. LNA includes bicyclic ribose analogue nucleotides producing a dramatic increase in binding affinity: up to +7 °C per modification when binding RNA [37] [38]. A large number of additional promising analogues of LNA have been synthesized with similar hybridization properties as LNA, but greater nuclease stability [39]. tcDNA is another promising constrained nucleotide for therapeutic splice-switching applications due to their preferred localization to the nucleus [40] and the treatment of Duchenne muscular dystrophy (DMD) in particular [41]. Two families of modified therapeutic ONs are based on structures that diverge completely from the natural nucleic acids: PMO and PNA, both of them showing increased bonding affinity, in part because of their neutral character. Phosphorodiamidate morpholino oligomers (PMO) have shown significant application as splice-switching ONs, in particular Eteplirsen has been FDA approved for DMD treatment [42]. To date, peptide nucleic acid (PNA) has not attained clinical trials, nevertheless, recent studies report the therapeutic potential of PNA as antibacterial compounds and anticancer drugs [43].

Due to this growing attention paid to ONs and the increasing number of clinical trials in which they are involved, in this review paper we describe the state of the art of ON production. In particular, we critically discuss both the upstream, based on solid-phase synthesis or on recombinant technology, and the downstream part, largely based on the use of chromatography. Considering the first positive results as therapeutic agents, it is reasonable to envision that in the near future relatively large production facilities will be needed. This will probably require the use of more efficient continuous technologies, replacing the current ones based on batch operation (e.g., [44]. Indeed, this situation reminds the recent development of monoclonal antibodies (mAb) as therapeutic agents. In this case, in the first 15 years after the approval of the first mAb, orthoclone OKT3 in 1986, only 15 mAbs received approval for clinical use and more

than 50% of them were subsequently removed from the market after a few years [45]. The turning point for the burst in the number of mAbs admitted to the clinical trials was the realisation of efficient production and purification processes. This, combined with the successful therapeutic activity of mAbs in the treatment of many pathologies, motivated the intensification of the research activity in this class of therapeutics [45]. Accordingly, one of the objectives of this review is illustrating how continuous chromatography, and in particular Multi Column Solvent Gradient Processes (MCSGP) can play a significant role in the transformation of ONs manufacturing, which would enable a sustainable scalability of their production to satisfy the market demands.

2. Synthesis of ON-based therapeutics

2.1 Solid-phase synthesis

The clinical success of ONs is mostly due to the possibility of assembling different sequences of a wide range of chemically modified ONs by a single fully automated solid-phase synthesis platform. The key steps of this synthetic approach based on the phosphoroamidite chemistry, *i.e.* deprotection, coupling, sulfurization/oxidation and capping, as schematically sketched in **Figure 2**, are common to most of the chemically modified therapeutic ONs. These include 2'-*O*-methoxy (2'-*O*-Me), 2'-*O*-(2-methoxyethyl) (2'-*O*-MOE) and 2'-F oligoribonucleotides, locked nucleic acid (LNA), tricycle-deoxyribonucleic acid (tc-DNA) and unnatural L-RNA ONs (also known as Spiegelmers) [46], in both phosphorothioate (PS) and phosphate (PO) linkage (**Fig 1A**).

In the phosphoroamidite approach, the synthesis is performed using solid supports where the chain grows in the 3' to 5' direction [47]. In the past, controlled-pore glass (CPG) was the solid support of choice. Today, due to the large-scale needed in the production of therapeutic ONs, high-loading polymeric solid supports are generally preferred. Among the latest generation of polystyrene supports, it is worth mentioning Primer Support 5G from GE that can be loaded up to 350 µmol/g, allowing ON synthesis at scales up to 750 mmol/reaction. Deprotection (or deblocking) is the first step of the synthesis cycle and it is accomplished in acidic conditions (3-10% dichloroacetic acid in toluene) with the removal of the protective group (4,4'-dimethoxytrityl group, DMT) from the support-bound nucleoside. The correct exposure time to acidic conditions during this step is important in determining the final crude oligonucleotide purity and yield. Overexposure results in depurination (adenosine primarily) [48], whereas too short detritylation times lead to incomplete deprotection with a consequent increasing fraction of ON sequences that lack in a single nucleotide (the so-called n-1 impurities) [49] [50] [51].

Next, as shown in **Figure 2**, the ONs are assembled through the coupling step of activated phosphoroamidites. Normally, for large scale synthesis, 1.5-2.0 equivalent excess of activated phosphoroamidite is required to obtain a coupling efficiency larger than 99% for each cycle. The activation of amidites is typically achieved using either a 1*H*-tetrazole-type (usually 5-(Benzylthio)-1*H*-tetrazole (BTT) or 5-(Ethylthio)-1*H*-tetrazole), or an imidazole-type (usually 4,5-dicyanoimidazole (DCI)) activator [52]. Sulfurization or oxidation follows the coupling. In this step, the reactive phosphite triesters are converted into more stable PS or PO triesters. The sulfurization reaction is fast and efficient (PS conversion greater than 99.9%) with freshly prepared phenylacetyl disulphide (PADS), but impurity formation has been detected with aged PADS solutions [53]. The final step in each synthesis cycle, referred to as capping, is used to silence by acetylation any terminal nucleoside that failed to react, so as to minimize n-1 impurities. Despite the phosphoroamidite synthesis has experienced a decisive improvement in the last years to satisfay the ON market requirements, several by-products are still associated with this synthetic route [54] [55]. To improve the separation of the desired ON from capped failure sequences, the ON is generally synthesized with its 5'-DMT protective group in place. Once the synthesis is complete, the support-bound ON is generally processed to remove base and phosphorothioate/phosphate protecting groups, and to cleave ON from the solid support. This is achieved through a two-step basic treatment, which avoids the addition of acrylonitrile to the *N*-3 position of thymine [56].

Phosphorothioate is probably the most used backbone modification. Usually, PS substituted therapeutic ONs are random mixture of stereoisomers (up to 2^n diastereoisomers) because PADS sulfurization creates in the achiral phosphitodiester a chiral phosphorothioate center with no stereochemical control. A scalable solid-phase synthetic process that yields therapeutic A-ONs having high stereochemical and chemical purity has been recently realised using stereopure amidites [57]. The influence of the stereochemistry of PS modifications on the pharmacological properties of ONs has also been investigated [58].

In addition to the widely used phosphoroamidite approach, several alternative oligonucleotide chemistries have been developed such as, morpholino (PMO), peptide nucleic acid (PNA) and N3′-P5′ thiophosphoramidates ONs (NPS), as schematically sketched in Fig 1B. Phosphorodiamidate morpholino ONs (PMO) contain modified morpholine rings, sewed together with phosphorodiamidate linkages and standard nucleobases. They are prepared on a solid-support using a two-step cycle process that consists of deprotection (detritylation) and successive coupling of the chlorophosphoramidate-activated monomers [59]. PNA is a modified

oligonucleotide with a peptidic backbone instead of a phosphoribosyl backbone. Solid-phase synthesis is performed employing FMOC chemistry [60]. Two different kinds of solid support can be used: a peptide amide linker (PAL) resin or a xanthen alkonic acid (XAL) resin. A typical synthetic cycle consists of three steps (deprotection, coupling and capping) where the coupling occurs between an activated acidic monomer and the growing PNA chain. N3′-P5′ phosphoramidate ONs (NP), present each 3′-oxygen replaced by a 3′-amine group in the sugar. The solid-phase synthesis is performed in the 5′ to 3′ direction using activated 3′-tritylamino-5′-*O*-phosphoroamidite monomers [61].

2.2 Recombinant technology

Although the most established technology for the production of oligonucleotides is the solid-phase phosphoramidite synthesis [62], some authors argue that synthetic ONs are very different from the ones produced naturally by the cells [15] [63]. As an example, RNAs transcribed from the genome of cells do not typically contain any modification in the ribonucleotides and have only few post-transcriptional modifications (such as methylation, acetylation and hydroxylation) [63]. This is in contrast to synthetic ones which contain a panoply of different modifications. These alterations have some advantages, such as increasing half-life of the drug, however they can lead to immunogenic responses. On the contrary, natural post-transcriptional alterations, being more similar to cellular RNA molecules, can depress immune response [63].

This justifies the interest in the biological production of ONs, aside to the solid-phase synthesis. However, it is worth mentioning that the direct expression of the intended ON in microorganisms has only been achieved with very low yield, due to the abundant presence of RNases which attack and degrade heterogeneous RNAs present in the cells [15]. This has motivated researchers to find valuable alternative strategies to produce ONs through recombinant technologies. These include, for example, t-RNA scaffolds, in which the recombinant RNA to be produced is masked as a natural RNA of the cell, thus enabling its expression in fast-growing bacteria such as *Escherichia coli* [64] [65]. An alternative strategy is the stabilization of the RNA to be produced through the use of p19, a protein expressed by the plant viruses *tombusviruses* that possesses a strong affinity and selective biding to siRNAs. The stabilization offered by p19 has been exploited for the production of siRNAs in *E. coli*. These natural siRNAs showed exceptionally high efficiency towards the knockdown of the target gene when used to transfect mammalian cells [66]. Other approaches make use of rRNA [67] or optimal ncRNA [15] scaffolds. These techniques have been implemented with some degree of success [68], even in diverse bacteria [69], leading to significant yields and reducing production costs [63], very significant for the case of one of the

most expensive drug classes on the market [70]. This is due to the lower costs associated with fermentation, when compared to synthetic synthesis via nucleoside phosphoramidites oligomerization [63]. In addition, Pereira et al. [69] produced pre-miR-29b *via* fermentation using *Rhodovulum sulfidophilum*, which was able to excrete to extracellular medium the therapeutic ON, leading to a concentration of 182 µg/L in the extracellular media after 40 h of fermentation. The obtained product is mostly free from the attack of RNAases and endotoxins, thus improving the recovery yield.

Another interesting study by Ho et al. [68], reports a novel strategy for the productions of ncRNAs based on a more stable ncRNA carrier, which improves the expression yield of the oligonucleotide of interest up to 80% of the total RNA. In addition, the tRNA scaffold method developed by Ponchon et al. [65] has been shown to produce RNA at the scale of milligrams per litre of culture – a rather interesting level for biopharmaceutical production. Furthermore, since the scale-up procedures for fermentation bioreactors are well established [71], these processes have the potential to be readily scaled at the commercial scale.

From a more visionary perspective, it is worth pointing out that the bio-production of ONs would be very similar to that of therapeutic proteins [63]. Nowadays, the vast majority of proteins for clinical applications are recombinant proteins, produced *via* fermentation due to the assurance of correct folding and post-translational modifications and are not synthetized *in vitro* [63]. In this context, the adoption of continuous processes, and in particular perfusion bioreactors as the production unit, is getting more and more popular with respect to the classical batch technologies. Indeed, perfusion bioreactors provide a key step in the intensified and continuous integrated manufacturing of therapeutic proteins and in particular of mAbs [72]. We expect also for the synthesis of ONs a similar technological evolution, as soon as these drugs will achieve significant success in relevant therapeutic indications.

3. Analytical characterization of oligonucleotides

The use of ONs as therapeutics requires an accurate quality control during the entire production process. As discussed above, many impurities may be formed during their synthesis, including the so-called shortmers (*n-1; n-2;* etc.), longmers (*n+1; n+2;* etc.) and other by-products caused by deamidation, depurination, deprotection failure or adduct formation [73]. Therefore, sensitive and selective analytical methods, able to separate the target molecules from impurities, are needed for the assessment of purity of both crudes and final active pharmaceutical ingredients

(APIs). High performance Liquid Chromatography (LC) is indeed the technique of choice for this.

Since ONs can bear an elevated number of negative charges depending on their length, the most effective elution modes are ion-pair reversed-phase liquid chromatography (IP-RPLC), anion exchange (AEX) LC, hydrophilic interaction liquid chromatography (HILIC) and mixed-mode LC [74]. Detection of ONs is usually performed through a UV detector, typically at 260 nm, where they exhibit very strong absorbance by their heterocyclic ring. The use of fluorescence detection has also been reported [75] [76]. Obviously, LC interfaced to mass spectrometry (MS) is employed for detection of trace impurities, as well as for sequence determination and structure characterization [77].

In the following, the most recent results and applications in the field of analytical chromatography are discussed. For the sake of convenience, the information has been organized based on the elution modes listed above. For a more complete treatment of the chromatographic techniques employed for the characterization of ONs, the reader is also referred to other recent reviews on the subject [78] [74] [79] [77] [73].

3.1 Ion-pair reversed-phase liquid chromatography (IP-RPLC)

IP-RPLC represents the most widely used technique for the analysis of ONs [73]. Suitable counterions, typically quaternary alkyl ammonium compounds, are added in the mobile phase. The exact mechanism of ion-pairing has been subject of debate for several decades. Essentially, there are two possible theories. The first one hypothesizes that the formation of ion-pairs between charged analytes and counterions occurs in the mobile phase. Then, neutralized ion-pairs are adsorbed on the hydrophobic stationary phase. Since the sequence of nucleobases present in the structure of ONs determines their hydrophobicity, it also governs their chromatographic retention. For DNA-based ONs, this increases in the following order: C < G << A < T [80] [81]. Based on a second theory, alkyl counterions are firstly adsorbed on the stationary phase. This creates a sort of dynamically charged surface, which provides the stationary phase with an ion-exchanger character, thus allowing the separation of analytes based on their charge. Most likely, the separation of ONs under IP-RPLC is due to a combination of these mechanisms [74], where both hydrophobicity and charge play a role.

The first ion-pairing reagent used for the separation of ONs has been triethylammonium acetate (TEAA) [82]. Li and co-workers report the separation of a siRNA consisting of a 21-nucleotide

sense strand with one PS stereocenter and of a 23-nucleotide antisense strand containing two PS stereocenters by using TEAA in the mobile phase. siRNA duplex diastereomers were efficiently separated on cyano columns, while Bridged Ethylene Hybrid (BEH) C18 and BEH phenyl columns gave better results for the separation of related single-stranded siRNA stereoisomers. Resolution of diastereoisomers improved by increasing the temperature over that required for annealing siRNA [83]. Interestingly, it was shown that the use of shallower gradients and lower triethylammonium concentration increased the stereoselectivity of PS pentameric oligonucleotide diastereoisomers, separated on a XBridge C18 column [84]. Among structural factors affecting selectivity of this class of ONs, it is worth mentioning the type of nucleobase, on the one side, and the position of modified linkage, on the other. Indeed, it has been reported that diastereoselectivity is higher for cytosine-based pentamers (compared to thymine-based ones) and when substitution occurs in the centre of the pentamer, instead that at its end [84]. Other alkylammonium compounds have been investigated, including tributylammonium acetate (TBAB), hexylammonium acetate (HAA) and triethylammonium bicarbonate (TEAB). Moreover, the combination of triethylamine (TEA) with hexafluoroisopropanol (HFIP) is particularly suitable for LC-MS separations since HFIP not only stabilizes pH but also enhances the MS signal [74, 85] [86] [87]. Thanks to its volatility (much higher than that of, e.g., acetate), HFIP can be efficiently evaporated from droplet surfaces at the electrospray ionization (ESI) source. Due to its unique ion-pairing properties, which allow to improve resolution and selectivity of separation, TEA/HFIP is the preferred mobile phase for the separation of ONs also with other kinds of detection (especially, UV or fluorescence).

Alternative types of alkylamines have been investigated as mobile phase modifiers in combination with HFIP. Even if the chromatographic selectivity can be very similar, the concentration of alkylammonium ions can have a strong impact on the separation of different ONs, including DNA and RNA. It was demonstrated by TEM and dynamic light scattering experiments that when the alkylamine concentration is larger than 20 mM, alkylammonium ions form micelles in the mobile phase [88]. Under these conditions, therefore, micellar chromatography controls the retention mechanisms. When, on the contrary, this concentration is lower than 20 mM, the ions remain more strongly bound to the stationary phase. Accordingly, the retention mechanism is that typical of ion-pair chromatography.

Alkylammonium ions (acetate as counterions) have been recently considered for the LC-MS separation of product-related ON impurities, in particular positional isomers. The use of small alkylamines such as propylamine and isopropylamine provided sharper peaks and better

resolution of impurities in the analysis of phosphate diesters, over the more commonly employed TEA, TBA and HA (see **Figure 3**) [89]. However, when used to separate the closed-related impurities of PS ONs, small alkylamines lead to severe peak broadening. The replacement of the phosphodiester linkage with a PS moiety is a common strategy used to increase the hydrolitic stability of the ONs, but this modification introduces an additional chiral phosphorus centre in the molecule that multiplies the number of stereoisomers. It is important to point out that separation of PS close-related impurities can be very challenging also in this mode of chromatography and that desulfurization of thiophosphate linkages into their phosphate diester form is often required [89].

Several attempts have also been made to replace HFIP with other fluoroalcohols. Basiri et al. found that the MS signal of PS ONs can be enhanced when a combination of N,N-dimethylcyclohexylamine (DMCHA) and 1,1,1,3,3,3-hexafluoro-2-methyl-2-propanol (HFMIP) is used as mobile phase [90]. The enhancement was significantly stronger than with any combination of HFIP and ion-pairing reagents. It is also important to point out that HFIP and HFMIP produce significantly different mass spectra, due to different charge-state distribution-patterns, for ONs [91]. In addition, HFMIP is characterized by more pronounced desalting ability with respect to HFIP, which usually allows to obtain mass spectra that are simpler to interpret. From the analysis of different ON samples, by using different combinations of alkylamine and fluoroalcohols, Liu et al. concluded that the use of hexylamine with either HFIP or HFMIP is more suitable for small ONs, while tripropylamine in combination with HFMIP has to be preferred for large synthetic ONs [91].

In the vast majority of cases, C18 stationary phases are employed for the separation of ONs in IP-RPLC [74]. Recently, other adsorbents have also been considered. These include C18 with polar end-capped groups [92], pentafluorophenyl-based stationary phases [86], as well as styrene-divinylbenzene [93] and phenyl-based resins [94]. In particular, π-π interactions, typical of phenyl-based stationary phases, have been demonstrated to increase retention of ONs [94]. In order to reduce mass transfer and improve the separation of ONs, columns packed with particles of very small diameter (below 2μm) or core-shell particles have been used. Separations of ONs are indeed characterized by very low mass transfer. As an example, core-shell particles have been recently employed for the rapid determination of high-resolution fingerprints of single-stranded and double-stranded RNA [95]. On the other hand, the effect of particles pore-size on mass transfer has not been systematically investigated so far. Monolithic columns are also attractive for the separation of ONs [96] [93] [97]. Interestingly, Qiao et al. have demonstrated that hairpins

are always less retained than random coils on monoliths, while no retention was observable with particle-packed columns [96]. This has been attributed to the fact that hairpins are more easily deformed into particle-packed columns, leading to more complex retention behaviours.

3.2 Ion-exchange chromatography (IEX)

Anion-exchange (AEX) chromatography with UV or fluorescence detection represents a very useful technique for the separation of ONs, which bear several negative charges. AEX stationary phases possess positive charged groups on their surface (e.g., quaternary ammonium groups). Negatively-charged ONs are retained through electrostatic interactions. Separation is controlled by modifying the amount of competitive anions present in the mobile phase. Typical mobile phases consist of NaCl in phosphate buffer or Tris. Longer ONs (with a larger number of charged sites) are more retained on AEX stationary phases than shorter ones. Therefore, AEX is very efficient for the separation of N-x deletions. On the opposite, other chromatographic modes must be employed for the resolution of ONs with very similar structures [74]. Monoliths have also been tested as support for AEX. A methacrylate-based monolithic column, for instance, was shown to be suitable for the separation of both single- and double-stranded DNA molecules with a mobile phase made of NaCl in Tris-HCl buffer (20 mM) [98].

3.3 Hydrophilic interaction liquid chromatography (HILIC)

The first attempt of separating ONs in HILIC conditions dates back to 1990, when Alpert successfully separated a mixture of homo-ONs [99]. He proved that retention was influenced by the buffer concentration in the mobile phase. Elution was obtained by using shallow salt gradients. This technique, after about twenty years when it has been considered only sporadically [100] [101] [102] [103] [104], has been recently reconsidered as an effective tool for the separation of charged ONs. HILIC offers in fact a viable alternative to IP-RPLC, which often exhibits low sensitivity when coupled to MS, due to signal suppression in the presence of ion-pairing reagents. Studzińska et al. systematically investigated the influence of different factors, including mobile phase salt type and concentration, on the separation of ONs in HILIC-ESI/MS [105]. They found that retention of PS ONs is affected by the number of nucleotides in the sequence and the percentage of nitrogenous bases. Moreover, the elution strength was higher when ammonium formate was used, compared to ammonium acetate. HILIC allowed to reach significantly lower limit of quantifications compared to IP-RPLC. On the other hand, if the objective is to resolve very complex multi-component mixtures, IP-RPLC remains the method of choice.

Interesting examples of fast separations of unmodified ONs (less than ten-minute analysis time) were reported in HILIC by using a Zorbax column, with 10 mM TEAA buffer and acetonitrile as mobile phase [106]. The same approach allowed also to resolve sequence isomers and chemically modified ONs such as phosphorylated, phosphorothioated, and fluorescently labelled species. Fast separations of deoxynucleic acid oligomers, modified and unmodified oligoribonucleotides, and PS DNA ONs have been performed on diol columns by using ammonium acetate buffer and acetonitrile as mobile phase [107].

Finally, as mentioned before, the very efficient desalting occurring under HILIC conditions allows to obtain simpler mass spectra than in IP-RPLC [106]. Therefore, HILIC represents a sensitive and robust alternative to IP-RPLC with significant potential for both qualitative and quantitative analysis.

3.4 Mixed-mode chromatography (MMC)

Since ON mixtures generally contain a wide variety of impurities, a single separation mode often is not sufficient to separate all of them. For example, separation of *N-x* deletions, which represent the most abundant impurities produced from the failed addition of nucleotides during the synthesis, can be efficiently achieved by means of AEX. On the other hand, AEX is not suitable to detect depurinations, base substitutions, PS diastereoisomers and other species with subtle changes on their sequence. In these cases, the complementary IP-RPLC mode is preferred. The recently developed mixed-mode stationary phases combine reversed-phase and ion-exchange properties in the same column, representing a very attractive alternative for the separation of ONs [108]. The mobile phase is pivotal in determining the behaviour of these columns. Indeed, depending on the composition of the mobile phase either hydrophobic interactions or ionic-exchange prevail. In the case of Scherzo mixed-mode columns, for instance, hydrophobic interactions have been demonstrated to be dominant when the mobile phase is made of ammonium acetate or triethylammonium acetate. On the other hand, if operated under NaCl or NaBr salt gradients, these columns exhibited a completely different retention trend for N-x deletions and also allowed for the resolution of isomeric ONs [109]. pH of the mobile phase also plays an important role to control retention and selectivity. For example, in the case of alkylamide stationary phases, as reported in Ref. [110], when pH is buffered at 6 or 7, the negative charges on the stationary phase are not efficiently shielded by the salts. ONs are therefore repelled from the surface and retention is dominated by hydrophobic interactions. On the contrary, when pH is around 5 or below, electrostatic interactions are predominant. Very recently, Lämmerhofer's

group synthesized two novel mixed-mode stationary phases, by means of a stable polymer coating strategy, that allowed limiting the bleeding phenomena often observed with prior brush-type RP/WAX congener with bifunctional siloxane bonded ligand. In a second version, the residual thiols on the polymeric RP/WAX were oxidized to sulfonic acids to obtain a RP/WAX/strong cation exchanger (RP/WAX/SCX) stationary phase. These groups act as surface anchored counterions, shifting the still net positive surface charge to lower ζ-potentials. This modification allowed to accelerate the separation of negatively charged ONs, with a reduction of the total analysis time by about 50% [111].

4. Preparative chromatography for the purification of ONs

In order to promote the commercialization of ONs as therapeutics, they need to be produced in sufficient amount firstly for their bioactivity to be characterized, then for preclinical and clinical trials and, eventually, for the transition to the market [112]. Efficient technologies for the preparative purification of significant amounts (mg to g) of target ONs from production impurities (such as shorter nucleotidic chains, aggregates, small abortive transcripts, etc.) are therefore urgently required. The choice of the purification method relies on the chemical features of ONs, especially their modifications and applications. For instance, it is very well known the importance of the tertiary structure of these molecules in influencing their pharmaceutical activity. If the purification method is excessively harsh, denaturation of ONs can occur, requiring a refolding protocol in order to recover the original structure and functional activity.

Two are the reference techniques for the downstream processing of ONs, namely preparative chromatography and polyacrylamide-gel electrophoresis (PAGE). Our anticipation is that, thanks to their larger versatility, greater flexibility, ease of automation, scaling-up and continuous production, LC applications will become the preferred choice for the preparation of therapeutic ONs. Therefore, PAGE-based purification methods will not be discussed any further in this review. The interested reader can refer to specific reviews and papers on this subject [113] [114] [112].

In most cases, ON purifications via LC are based on the methods already discussed before for analytical purposes, including IEXLC, RPLC and IP-RPLC. On the other hand, only a few examples of size-based separations via Size Exclusion Chromatography (SEC) have been reported. Here the problem is that usually the required purity cannot be met in a single step and therefore other purification strategies (e.g., extraction, other LC separations, etc.) need to be performed after SEC [114] [115].

4.1 Ion exchange liquid chromatography

As mentioned above, ONs very often carry charges on their chains, so that ion-exchange chromatography is one of the preferred techniques for their purification. Stationary phases usually bring tertiary or quaternary ammonium groups, and are used in gradient mode using salt as a modifier. ONs are separated based on the charge of PO groups or other substituents along the chain. Usually, the PO backbone carries a negative charge so that the full-length ON is the last to elute [116] [117]. Both weak and strong anion exchangers have been considered for the purification of ONs. Small abortive transcripts show a small negative charge, while larger transcripts exhibit a larger negative charge which increases with their size. Accordingly, plasmid DNA template has the largest negative charge while T7-RNA Polymerase has neutral isoelectric point (pI). These species have been successfully separated on a weak anion exchange resin, i.e., diethyl amino ethyl (DEAE)-sepharose, with the following retention times: small abortive transcripts < target oligonucleotide < plasmid DNA. This method allowed the direct purification of the crude mixture, without the typical preliminary extraction step with phenol [118]. On the other hand, DEAE-sepharose has been proven to be unsuitable for separating RNAs with different 3' ends. Among strong anion-exchangers, it is worth mentioning the MonoQ stationary phase [119]. It has been found that this stationary phase is able to separate ONs depending on their lengths, without the preliminary extraction step. Separation of ribonucleoside tri-phosphate (rNTP), short abortive transcripts and target RNA on IEX has also been investigated. However, the adopted high ionic strength and pH values can have a denaturing effect on ON structures, so that weak anion-exchange is to be preferred if the native RNA structure is to be preserved.

4.2 Reversed-phase liquid chromatography (RPLC)

RP-LC has also been considered for the purification of ONs. Since in RP-LC the adsorption is strongly related to the percentage of organic modifier in the mobile phase, gradient operation is needed in order to modulate the selectivity of the chromatographic system [120] [121]. Good resolutions are obtained only with shallow gradients, at the cost of significantly longer process time. This technique is particularly suitable for ONs modified with hydrophobic groups or with fluorescent dyes. The drawback is that this notoriously leads to the biomolecule denaturation, thus compromising its bioactivity [120] [122].

4.3 Ion pair reversed-phase liquid chromatography (IP-RPLC)

In the case of IP-RPLC, usually an alkyl amine is added to the mobile phase as ion pair reagent.

The involved complex adsorption mechanism depends on the charge of the ONs, the length of the alkyl substituents and the percentage of organic modifier in the mobile phase [81] [123]. The larger the hydrophobicity of the mobile phase and the ion-pairing reagent, the higher the retention. Therefore, longer alkyl chains in the ion-pairing buffer lead to longer retention times, while, on the contrary, if the fraction of organic modifier is increased, the analytes tend to leave earlier the column [122]. This technique is particularly suitable for the separation of RNA chains with only slight differences in the sequence and with length no larger than 60-mers.

5. From batch to continuous chromatography

As we discussed above, the literature on the preparative purification of ONs is still very scarce, and in addition it is strictly limited to single column (batch) chromatography. In this operation, it is well known that, in order to achieve the desired purity of the target molecule, it is necessary to properly narrow the collection window. This typically implies low recovery values that in the case of ONs may be as low as 40%, and in many cases even lower. To avoid wasting the target product, the overlapping regions in the eluting stream (where the target component is still present but with an excessive amount of impurities) are manually recycled and reprocessed. This is a very labor-intensive activity decreasing tremendously the productivity of the process, which in turn becomes inacceptable for commercial standards.

This problem, referred to as the purity-yield trade-off, is an intrinsic feature of single column batch operations, and can be alleviated introducing multicolumn or continuous chromatography (e.g., [44]). By simultaneously operating a series of (identical) chromatographic columns properly connected through several switching valves, this technique permits not only the continuous and automatic operation of the unit, but also to mimic the countercurrent movement of the stationary phase in the opposite direction to that of the eluent stream. This leads to significant improvements in the efficiency of the process, which can be quantified in terms of a significant recovery increase, with the same purity, with respect to single-column operations.

In particular, for the purification of mixtures of ONs, the so-called Multicolumn Countercurrent Solvent Gradient Purification (MCSGP) process, based on only two twin columns, seems particularly suitable [124] [125] [126] [127]. This technique, previously used for the purification of peptides, proteins and monoclonal antibodies, has been recently used for the purification of a typical mixture of ONs [128]. As mentioned above, it was found that, at fixed purity (92% in this case), MCSGP allowed to increase the yield from 55% to more than 90% with respect to single column operation. The yield increase in MCSGP is due to automated internal recycling of

the product-containing impure side-fractions. **Figure 4A** shows a single-column chromatogram of anion-exchange purification using a salt gradient elution in NaOH-based buffers. The plot of the purity values reveals that the process is suffering from a yield-purity trade-off as the purity is below the required threshold (92%) in both the peak front and the peak tail, as indicated in the figure. The MCSGP process was designed based on the preparative chromatogram [126] and operated over multiple cycles in a cyclic steady state. **Figure 4B** shows a superimposition of the 5 cycles operated with the MCSGP technology. This confirms that the chromatograms are matching, indicating that the process has reached cyclic steady state. In cyclic steady state, a product of equal concentration and purity is withdrawn from cycle to cycle. In **Figure 4B,** also the impure side fractions selected for automated internal recycling are highlighted. A side-by-side comparison of single-column chromatography and MCSGP is shown in the yield-purity chart of **Figure 5**, indicating that the yield of MCSGP was about 50% increased (from 55% to > 90%) compared to the single column reference run. Therefore, MCSGP represents a scalable process solution for ON purification with equipment available for both process development on bench scale and pilot or commercial scale under GMP standards.

6. Conclusion

The FDA approval of the first ON Fomivirsen in 1998 marked the raise of a new class of macromolecular drugs holding promise in the treatment of severe diseases that were previously considered untreatable.

However, after more than 20 years of research and of good pre-clinical results confirming the expectations, only 9 products eventually reached the market. This is principally imputable to poor delivery, uptake and bioavailability of ONs *in vivo*. On the other hand, a relevant aspect that may explain the delay in the FDA approval for several ON chemistries is the lack of economical and efficient production protocols of ONs with desired features and purity.

Indeed, the upstream marked the most important advances over the years. Nowadays, the phosphoroamidite-based solid-phase synthesis is a reliable strategy for the preparation of chemically modified ONs with reasonable productivities. An automated synthesizer is now commonly used on 500-1000 mmol scale for kilo-scale production of therapeutic ONs. In addition, the evolution of continuous bioreactors, driven by the increasing demand of peptides and mAbs, is nowadays leading to the reconsideration of the synthesis of ONs through recombinant technologies. These show the advantage of a reduced immune response, even though careful synthetic protocols need to be designed to minimize the degradation operated by

nucleases. As a matter of fact, it can be concluded that different chemistries are required for different technologies, such as splice-switching, aptamer, siRNA, miRNA, etc.

A more delicate aspect in the ON production is the DSP. Only a few reports exist on the preparative purification of ONs and these are limited to the use of single column (batch) chromatography. The characteristic yield-purity tradeoff of this batch configuration evidently affects the whole process. A concerted effort in this field is therefore urgently required to cover the gap with the upstream. In particular, we envision that the application of multi column countercurrent chromatography can be an important breakthrough in the field as preliminary experiments using the MCSGP technology suggest.

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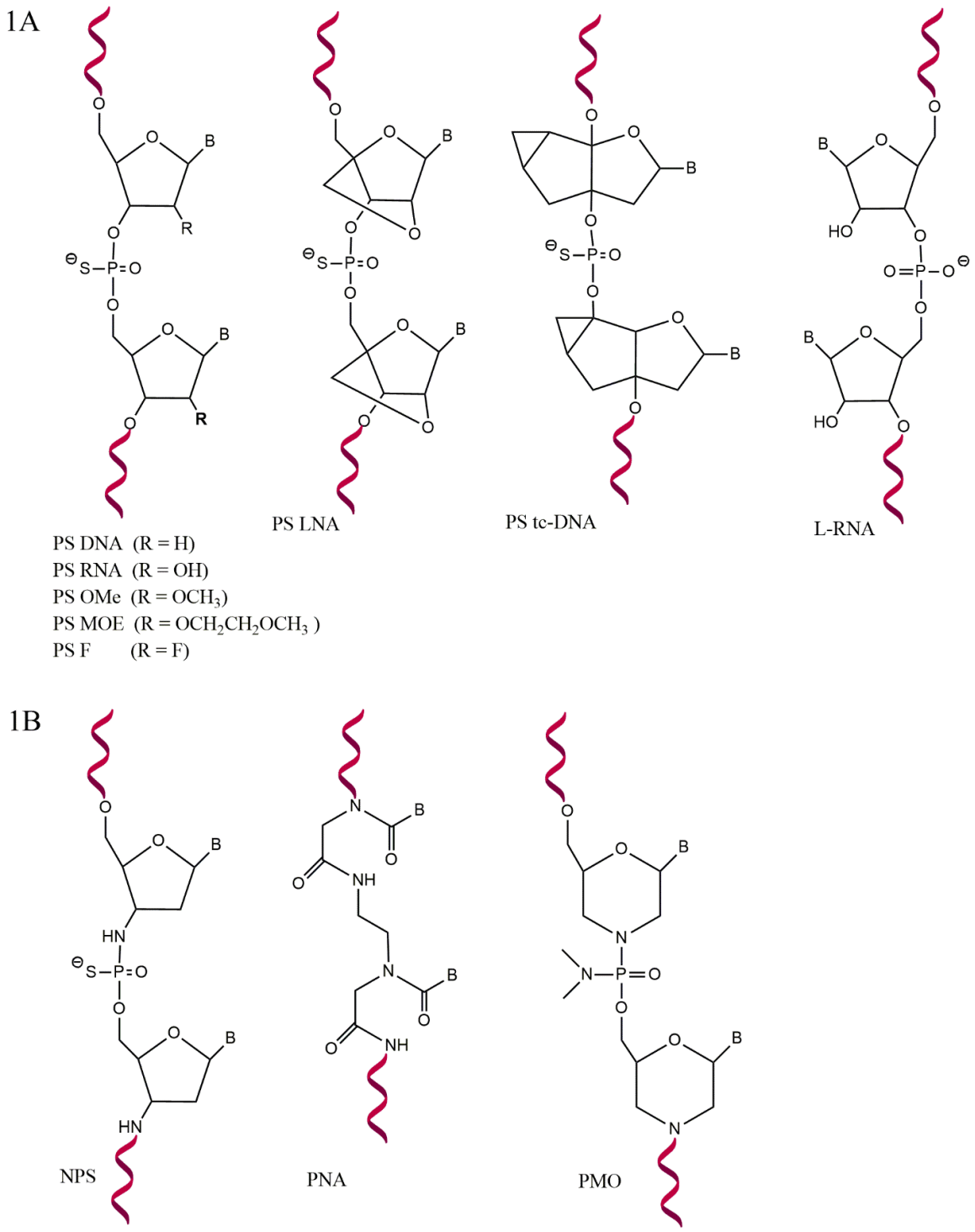

Figure 1: Chemical structures present in ONs currently employed in clinical or preclinical tests: 1A) ONs synthesized via the phosphoroamidite approach; 1B) ONs synthesized through other approaches: N3′-P5′ phosphoramidates (PSA), peptide nucleic acid (PNA) and morpholino (PMO).

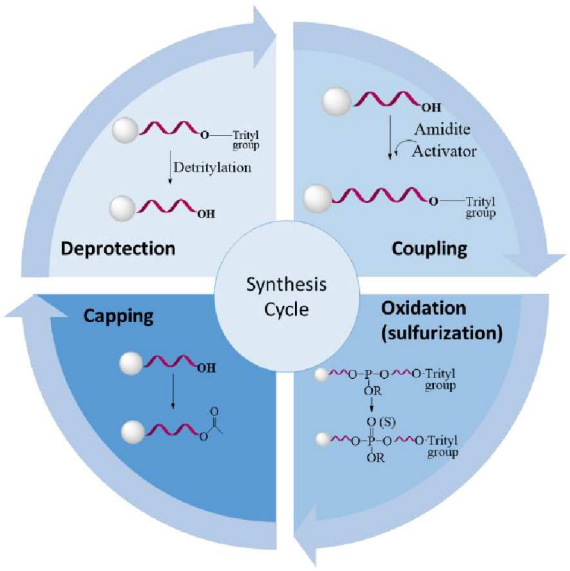

Figure 2: The four steps of the synthesis cycle using the phosphoroamidite approach.

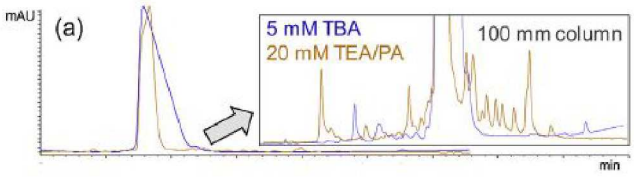

Figure 3: Chromatograms showing the IP-RPLC separation of a phosphate diester oligonucleotide using large (blue, tributylamine) and small (orange, mixture of triethylamine and propylamine) amines. Column: Waters XBridge C18 OST column (2.1 × 100 mm, 1.7 µm), 250 µL/min. Reproduced with permission from [89].

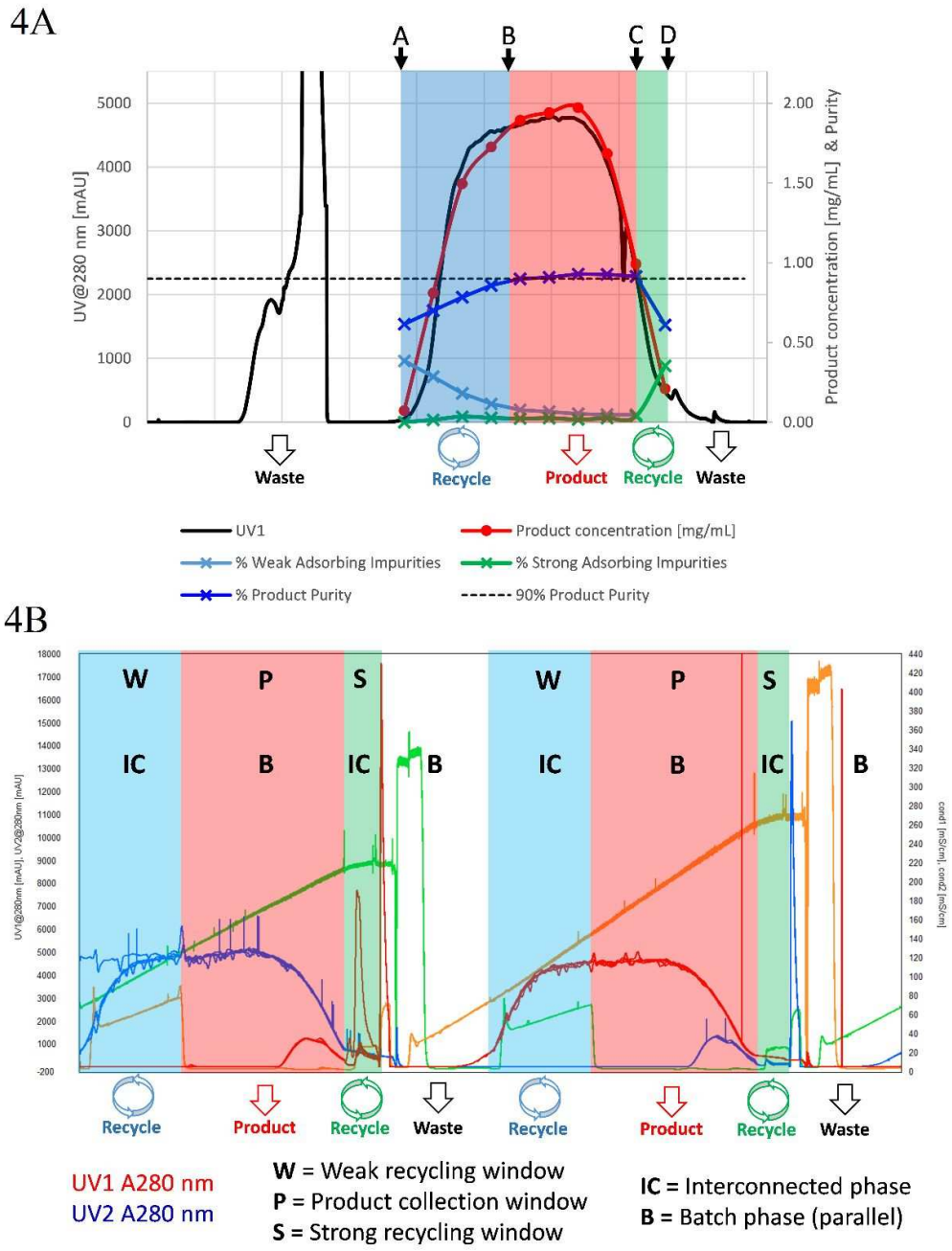

Figure 4: 4A) Preparative single column chromatogram and results from offline HPLC fraction analysis. Product purity and definition of the zones of the chromatogram selected for internal recycling (A-B in blue and C-D in green), and the product elution window (B-C in red), are also shown. 4B) Superposition of 5 cycles of a representative MCSGP run for purification of an oligonucleotide. It can be seen that the elution profiles change only slightly and that the product peaks are very similar. The first set of product elutions is from column 2 (blue), the second from column 1 (red). Reproduced with permission from [128].

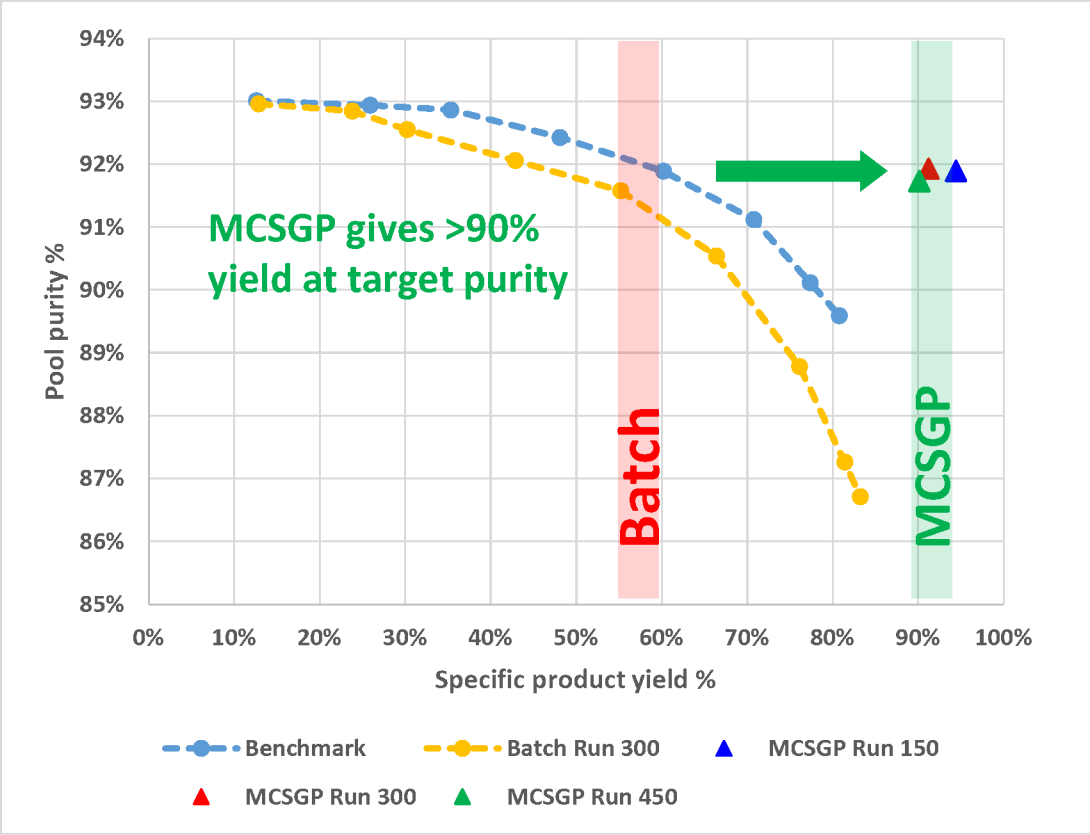

Figure 5: Pareto curve of the MCSGP (triangles) and the single-column batch reference run (circles). The data points of batch chromatography represent product pools of different sizes. MCSGP data points correspond to product pools of complete runs (5 cycles each). Reproduced with permission from [128].

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