Organic Solvent Nanofiltration (OSN): A New Technology Platform for Liquid-Phase Oligonucleotide Synthesis (LPOS)

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Abstract

Organic Solvent Nanofiltration (OSN) technology is a membrane process for molecular separation in harsh organic media. However, despite having well-documented potential applications, development hurdles have hindered the widespread uptake of OSN technology. One of the most promising areas of application is as an iterative synthesis platform, for instance for oligonucleotides or peptides, where a thorough purification step is required after each synthesis cycle, preferably in the same working solvent. In this work, we report a process development study for liquid-phase oligonucleotide synthesis (LPOS) using OSN technology. Oligonucleotide (oligo) based drugs are being advanced as a new generation of therapeutics functioning at the protein expression level. Currently, over one hundred oligo based drugs are undergoing clinical trials, suggesting that it will soon be necessary to produce oligos at a scale of metric tons per year. However, there are as yet no synthesis platforms that can manufacture oligos at >10 kg batch-scale. With the process developed here, we have successfully carried out 8 iterative cycles of chain extension and synthesized 5-mer and 9-mer 2'-*O*-methyl oligoribonucleotide phosphorothioates, all in liquid phase media. This paper discusses the key challenges, both anticipated and unexpected, faced during development of this process, and suggests solutions to reduce the development period. An economic analysis has been carried out, highlighting the potential competitiveness of the LPOS-OSN process, and the necessity for a solvent recovery unit.

Keywords: Organic solvent nanofiltration, OSN, membrane, oligonucleotide, liquid phase synthesis

1. Introduction

The discovery of RNA interference (RNAi), and its exquisite control of protein expression, has reinvigorated the consideration of oligonucleotide (oligo) based drugs as potential therapeutic agents¹⁻⁵. There are currently three oligo-based drugs approved by Food and Drug Administration (FDA): formivirsen (Vitravene), mipomersen (Kynamro), and pegaptanib (Macugen). The first two operate through anti-sense mechanisms, while the last is an aptamer. Following on from these successful market entries, over 100 oligo-based drugs are now going through different phases of clinical trials⁶.

One of the major challenges for the exploitation of oligo-based therapeutics, apart from the difficulties in drug delivery^{7, 8}, is the large-scale production of these complex molecules. As more drugs are developed which target a broader range of patients, it is anticipated that metric tons per annum of such drugs will soon be required^{9, 10}. Thus, there is a pressing need to develop economically viable processes to produce oligos at this scale¹⁰⁻¹².

The chemical basis of the synthetic methodology has remained essentially the same for the last 40 years. DNA and RNA are heteropolymers, with their mode of action defined by the sequence of the monomers. Therefore the synthesis employs step-wise addition of each nucleotide monomer, followed by separation of the reagent debris. The current state-of-the-art oligo synthesis platform is solid-phase oligonucleotide synthesis (SPOS)⁶. The key feature of SPOS is the use of a solid support to which one end (the 3'-end) of the growing chain is anchored. After immersion of the tethered oligo in a solution of reactive monomer, the solid support facilitates rapid removal of the excess reagents after each step, where the solid beads are simply washed with solvents. The growing chain on the solid support is then ready for the next round of chain extension reaction. To date, the versatile SPOS platform, with its high efficiency, has been able to meet the small-scale demands of research and there have been no fundamental changes to the SPOS process for the past 20 years.

Despite its clear advantages of easy purification and rapid synthesis, the general consensus with regards to scaling up the SPOS process is that it is very difficult for several reasons^{11, 13, 14}. First, the heterogeneous nature of the SPOS process leads to high mass-transfer resistance between the bulk solution and the support surface, and

introduces steric hindrance from the support. Thus SPOS requires a higher excess of the expensive monomers to drive coupling to completion than would be required in solution phase. Secondly, the intensity of SPOS is restricted by sub-maximal loading of the polymeric support to minimize interference between neighboring chains (particularly when preparing longer oligos), and thus maintain coupling efficiency¹⁵. Thirdly, the SPOS process is highly mass intensive at large scale⁶. For instance, to produce 1kg of 20-mer phosphorothioate oligos, approximately 4,000 kg of solvents, reagents, and water are needed¹⁶.

Another factor that is rarely considered in SPOS scale-up is the difficulty of intermediate analysis. On a large scale, where the loss of one batch is highly undesirable in terms of economics and operation time, sampling each cycle would be of great utility to detect failed steps when remedial measures can still be effected. However, representative sampling of solid phase from a large, packed-bed reactor is technically challenging, and in practice is not performed. Thus, product assay is again conducted only once the entire synthesis is complete, and in the event of one poor step the whole batch must be extensively purified, or even discarded. Despite the drawbacks, many significant breakthroughs have been made in SPOS scale-up recently, as reviewed by Sanghvi⁶.

Liquid-phase oligonucleotide synthesis (LPOS) has long been proposed as an attractive alternative large scale platform to SPOS¹⁷. Unlike SPOS, the homogeneous LPOS process does not suffer from mass-transfer limitations between the bulk solution and the surface of the support, allowing lower reagent excess. LPOS also occupies a lower reaction volume than SPOS because the inert interior of the solid support no longer needs to be accommodated within the equipment. However, although LPOS offers significant advantages over SPOS, it suffers the major drawback of the cumbersome downstream purification required to remove reaction debris after each iterative cycle.

In LPOS, the growing oligo is often anchored to a soluble support which is used to facilitate the downstream purification using techniques such as precipitation^{18, 19}, extraction²⁰, or membrane separation¹⁷. Bayer and Mutter¹⁷ first identified membrane separation as a key adjunct to liquid phase peptide synthesis for downstream processing. The same laboratory later applied this strategy to oligo synthesis using the obsolete

phosphodiester coupling strategy and diafiltering in *aqueous* solution²¹. Until recently, membranes could not effectively be employed for oligo purification because readily available polymeric membranes were not stable in organic solvents and lacked molecular discrimination. However, recent Organic Solvent Nanofiltration (OSN) membranes can now withstand harsh conditions, such as aggressive solvents [e.g. *N,N*-dimethylformamide (DMF) and *N*-methyl-2-pyrrolidone(NMP)] as well as the oligonucleotide reaction solvent (acetonitrile), while being stable over a wide range of pH, and resilient to both organic acids and bases²². More importantly, OSN membranes now have nano-separation capabilities^{23, 24}, discriminating solutes in the range between 100 and 2,000 Da.

Figure 1. Schematic of the LPOS-OSN process composed of 4 unit operations per cycle²⁵: a) chain extension; b) first membrane diafiltration to wash out excess reagents; c) deprotection of 5'-O using an acid to free the growing end; and d) second membrane

diafiltration to remove all the reagents except the growing oligos. [Reprinted with permission from reference 25, Copyright Wiley-VCH Verlag GmbH & Co].

This research group has previously explored liquid-phase peptide synthesis²⁶, in which the growing peptide chain was attached to a large mono-methoxy polyethylene glycol (mPEG-5000) support. Filtration of crude reaction mixtures through an OSN membrane allowed the excess reagents and impurities to pass, but the soluble peptide-PEG conjugate was retained by the membrane, and was thus purified for the next step. Building on this earlier experience, we have reported the application of OSN purification to LPOS, henceforth a process termed LPOS-OSN²⁵. Using the LPOS-OSN process, we have successfully synthesized 5-mer and 9-mer 2'-*O*-methyl phosphorothioate oligoribonucleotides. The synthesis was carried out using commercially available 5'-*O*-Dmtr-2'-*O*-methyl nucleoside phosphoramidite building blocks (Dmtr = 4,4'-dimethoxytriphenylmethyl), employing the standard coupling method²⁷.

The entire process proceeds in the liquid phase, as illustrated in general schematic Figure 1. A branched mono-disperse support was selected, on which three oligo chains could grow simultaneously, increasing the atom efficiency of the process. The first nucleotide monomer, N¹, was attached to the soluble support via a succinate linker that is cleaved at the end of the synthesis. To prepare an oligo of *n* non-identical monomers, the iterative growth cycle then requires two synthetic steps, with membrane purification after each, to be repeated up to the desired length: A building block (N^{*n*}-*OP*), bearing a temporary protecting group (*P*), is first coupled to the chain terminus of the growing oligo (Figure 1a); the temporary protecting group *P* is then removed (Figure 1c) to commence the next chain extension cycle. After each reaction, the excess reagents are washed through the OSN membrane (Figure 1b, d), in a process known as constant volume diafiltration, CVD. Two specialized terms routinely employed in this paper are *retentate* and *permeate*: These refer to the solutions retained by the membrane and permeated by the membrane, respectively, during a CVD.

In this work, we report the development of an LPOS process using OSN as the key separation technology. Typically, one of the main hurdles to the widespread applications of membrane technology is the long and circuitous process development period. There has been much effort in academia over the past decade to tap into the potential of OSN

technology, with some success. We have investigated some of the challenges encountered during the process development stage, both expected and unexpected, and proposed suitable solutions not only for this work, but for other potential OSN applications. In addition, this first pilot project for LPOS-OSN was used to provide enough practical experience to construct an economic analysis to assess the feasibility of scaling up the proposed process. It is shown that the LPOS-OSN process is highly competitive and scalable for large-scale oligo synthesis, and we assert that the LPOS-OSN process is a promising platform to make large-scale (>100 kg) synthesis of oligonucleotides a reality.

2. Experimental

2.1. Materials

Acetonitrile (MeCN), methanol (MeOH), *N,N*-dimethylformamide (DMF), dichloromethane (DCM), *N,N*-dimethylacetamide (DMAc), diethyl ether (Et₂O), ethanol (EtOH), and isopropyl alcohol (IPA), were HPLC grade from VWR, UK. Anhydrous solvents were prepared by storing the solvents over baked 4Å molecular sieves cooled under high vacuum (oil pump). Celazole® S26 polybenzimidazole (PBI) solution (26 wt% polymer in DMAc containing 1.5 wt% LiCl) was purchased from *PBI Performance Products Inc., USA*. Non-woven polyolefin Novatexx 2471 was from *Freudenberg Filtration Technologies, Germany*. The cross-linker α,α' -dibromo-*p*-xylene (DBX) was purchased from VWR. 5'-*O*-(4,4'-Dimethoxytriphenylmethyl) (Dmtr) 2'-*O*-methyl nucleoside phosphoramidite building blocks were purchased from *ChemGene Corp., USA* or *Fisher Scientific Ltd., UK*. *S*-ethylthiotetrazole (ETT, 0.25M solution in MeCN, *Proligo*), *N*-methylimidazole (NMI), phenylacetyl disulfide (PADS), pyridine, pyrrole and dichloroacetic acid (DCA) were bought from *Sigma-Aldrich, UK*. Polyethylene glycol (PEG) solutes were purchased from VWR, UK.

2.2 PBI 17DBX Membrane Fabrication & Testing Rig

Although several polymeric and ceramic membranes were screened for this work, only the preparation of PBI membranes is described here, as they reproducibly gave the desired performance (Please refer to Supporting Information for other membrane data).

PBI dope solutions were prepared by diluting the Celazole® S26 solution to 17 wt% with DMAc. Once a homogeneous solution had been obtained, it was cast onto polyolefin non-woven support using a casting knife (set to 250 μm). The supported film was quickly immersed in a deionized water coagulation bath for phase inversion. The resultant membrane was washed with IPA to ensure complete removal of water. To cross-link the polymer, the membrane was immersed in a well-stirred 3 wt% solution of DBX in MeCN and heated under reflux at 80 °C for 24 hours. The membrane was then removed from the cross-linking solution and washed with IPA thoroughly. Finally, the membrane was immersed in a PEG-400:IPA (1:1 v/v) impregnation bath for 4 hours, and then air-dried. This formulation of PBI membrane is referred to as PBI 17DBX (17 wt% PBI cross-linked with DBX). For further details of PBI membrane fabrication, readers are referred elsewhere^{28, 29}.

For membrane testing and purification of oligos, membrane discs (effective area of 51 cm²) were loaded into four cells connected in series. The diagram of the process rig is shown in Figure 2.

Figure 2. Membrane testing & diafiltration rig. Four membrane cells (effective membrane area of 51 cm² per cell) were connected in series ($N = 4$). The feed tank (stainless steel) was pressurized using nitrogen, and the system volume was maintained by constant addition of pure solvent back into the feed tank.

The feed tank was pressurized to the desired pressure (typically 10 bar) with nitrogen and the gear pump (*Michael Smith UK Ltd. GL Series*) provided a flow rate of 66 L.hr⁻¹. Before running preparative oligo purifications using PBI membranes, the membranes

were first characterized for rejection performance using a mixture of five linear PEGs (MW of 200, 400, 1000, 2000, and 8000 Da), 1 g.L⁻¹ of each dissolved in MeCN. For preparative oligo purifications, the rig was charged with the crude mixture and the total system volume was set to 0.4 L. The solvent was either neat MeCN, or MeCN mixed with varying proportions of MeOH (discussed in detail in the Results section). To balance the loss of system volume to the permeate, an equal volume of pure solvent was pumped into the feed tank using an HPLC pump (*Gilson 305, UK*) to maintain a constant level. The temperature of the rig remained constant at 21 ± 1 °C. Whenever necessary, samples were taken from both the permeate and the feed tank for HPLC analysis.

In CVD, the term *diavolume* is a convenient *time-like* dimensionless variable defined as the following

$$diavolume = \frac{J \cdot A \cdot t}{V_{system}} \quad (\text{Eq. 1})$$

where J represents the membrane flux (L.m⁻².hr⁻¹), A the membrane area (m²), t the filtration time (hr), and V_{system} is the volume of the system (L). Simply put, one *diavolume* means that the total permeated volume is equal to the system volume, which is maintained at a constant level.

This parameter is useful for comparing CVD efficiencies and performances, and will be used throughout this manuscript. For example, at the same final purity, a 10 diavolume CVD is more solvent efficient than a 20 diavolume CVD.

2.3 Overall Process & Oligonucleotide Synthesis Chemistry

Figures 3 and 4 summarize the overall process and chemistry schemes, respectively, for the LPOS-OSN platform developed in this work. The process is divided into three phases: initial loading of the support, the chain extension cycle, and release and purification of the final oligos. In the first phase, the first monomer of the chosen sequence (2'-*O*-methyl uridine) was conjugated to the branched soluble support [tris(octagol) homostar], after which the 5'-hydroxyl was deprotected to give mono-nucleosidyl homostar using classical batch chemistry (see section 3.3). In the second phase, the loaded homostar was passed through $n-1$ chain extension cycles until the

desired sequence was obtained ($n-1$ couplings are required to obtain an n -mer), using the rig shown in Figure 2. In the last phase, the oligo was deprotected and cleaved from the support, once more using classical solution phase chemistry, ready for final purification by ion exchange chromatography.

In the above scheme the repetitive chain extension cycle is implemented using liquid phase handling and membrane purification. This cycle consists of two synthetic steps: in the first pair of reactions (*coupling* of the exposed 5'-OH to reactive phosphoramidite **5**, and *thioylation* of the internucleotidyl linkage with PADS) the next monomer is attached, collectively known as *chain extension*; in the second step, known as *detritylation*, the 5'-*O*-(4,4'-dimethoxytiphenylmethyl) (Dmtr, or dimethoxytrityl) protecting group is removed from the 5'-terminus of the growing oligo ready for the next round of the cycle. After the chain extension step the crude tris(Dmtr-oligonucleotidyl) homostar was partially purified using membrane CVD to remove small molecules, and after detritylation the crude tris(HO-oligonucleotidyl) homostar was again purified by membrane CVD, this time removing all the monomer debris.

Figure 3. The LPOS-OSN platform: overall process flowchart. The process is split into three phases: initial loading, chain extension cycle, and final purification. Each phase of the protocol is detailed in the experimental section.

Figure 4. The LPOS-OSN platform: overall chemistry scheme for the same three phases.

The chemical synthetic procedures used in this study are described in full elsewhere²⁵. The methods are summarized briefly below. The preparation of the monodisperse octagol homostar **1** synthesis support was adapted from the literature synthesis³⁰. The term *homostar* used in this manuscript refers to the branched polymer support having three arms with a site on the end of each on which an oligo is grown. Homostar **1** was first condensed with 5'-*O*-Dmtr-2'-*O*-methyl-3'-*O*-succinyl uridine **2** using 2,6-dichlorobenzoyl chloride (DcbCl) and N-methylimidazole (NMI), then the 5'-hydroxyl was unblocked using dichloroacetic acid (DCA) and pyrrole to provide, after chromatographic purification, loaded support **3**.

Each chain extension reaction was slightly different due to the changing molecular weight and solubility of each substrate, but a general chain extension protocol is described below: The starting oligonucleotidyl homostar (*ca.* 1.2 g) was dissolved in a small volume of DMF (2-4 mL) under N₂ and co-evaporated from MeCN (3 × 20 mL). The next phosphoramidite monomer (4.5 eq., i.e. 1.5 eq. per 5'-OH) was added as a dry

solid, and the mixture was dissolved in 0.25 M ETT in MeCN (9 eq.). After stirring for 35 min, PADS (9 eq., i.e. 3 eq. per 5'-OH) was added, the volume was doubled with pyridine, and stirring continued for another 30 min. Notably, PADS was used as obtained, instead of keeping a solution of PADS in pyridine or 3-picoline to develop overnight.

An additional *capping* step is usually included in the SPOS cycle, using acetic anhydride plus *N*-methylimidazole (NMI) to block any unreacted hydroxyl groups remaining after coupling as acetate esters, and so prevent them participating in subsequent chain extension cycles. However, we were unable to detect any incomplete reaction by HPLC, even though analysis of our homostar supported oligos should be especially sensitive to incomplete coupling because of the 3-arm geometry. Since there is no requirement for mass transfer between the bulk liquid and a solid phase interface, there was reason to suspect that couplings to our fully dissolved supported oligo would be faster and more complete than SPOS couplings (see Introduction). Hence, the capping step was deliberately omitted to shorten the chain extension cycle.

After chain extension and the subsequent membrane CVD, the supported oligo was washed out of the membrane rig and the solvent evaporated. The residue was re-suspended in dry DCM to which pyrrole (2 vol%) then DCA (1 vol%) were added to effect *detritylation* (deprotection of the Dmtr protection group); upon addition of DCA the substrate dissolved and an intense orange color appeared which dissipated over the next 30 min. To ensure complete removal of all the 5'-*O*-Dmtr ethers the reaction was monitored by TLC, when partially deprotected intermediates appeared as a ladder of spots that turned orange in trifluoroacetic acid vapor. If the reaction did not approach completion in 15 min, a further aliquot of DCA was added; large oligos tend to buffer acidic solutions, slowing detritylation. Upon completion of the reaction, the reaction was quenched with a volume of pyridine equal to the total amount of DCA used (approximately equi-molar amounts); with longer oligos a thick precipitate formed at this point, presumed to be the product. The solids were re-dissolved when diluted into MeOH-MeCN, and this solution was then subjected to another round of CVD.

To effect *global deprotection* and cleavage from the homostar support, a sample of supported oligo was suspended in MeCN to which was added diethylamine (20 vol%).

After 30 min the mixture was filtered through a pad of cotton wool to remove any insoluble material, the solvent was evaporated *in vacuo*, and the residue was re-dissolved in conc. ammonia. The solution was heated at 55 °C overnight and the next day filtered once more. The filtrate was evaporated to dryness, and the residue co-evaporated from ethanol (3×). Finally the crude solids were triturated with MeCN to remove protecting group and support debris.

2.4 Membrane Purification – Constant Volume Diafiltration (CVD)

After the chain extension (section 2.3), the crude material was first diluted with either MeCN or MeOH-MeCN (250ml, 5-20% MeOH, depending on the solubility of the oligonucleotidyl homostar) and the rig (containing 150ml of the same solvent) was charged with this solution. The rig was then pressurized to 10 bar to commence CVD, and this continued until a total of 13-15 *diavolumes* had permeated, after which the retentate was washed out of the rig; the solvent was evaporated to provide a mass balance, and the residue analyzed by NMR, MS, and HPLC.

After detritylation (section 2.3), the crude suspension (in DCM, DCA, pyridine and pyrrole) was diluted with MeCN or MeOH-MeCN (trinucleotide onwards; 250 ml, 5-20% MeOH). To ensure that DCM did not damage the O-ring seals or membranes, the crude solution was concentrated on a rotary evaporator until the vapor pressure reached 20 mbar at a bath temperature of 30 °C, when DCM was assumed to have been removed and further evaporation stopped. The crude solution was returned to the rig, which was once more pressurized to 10 bar for a second round of CVD. This time, the solvent for the first 5 *diavolumes* contained 1 *vol%* pyridinium dichloroacetate (Py.DCA) in MeCN or MeOH-MeCN (trinucleotide onward), followed by 10 *diavolumes* with neat solvent. After CVD the purified retentate was washed out of the rig, and the solvent evaporated. Finally, although it probably does not interfere in the subsequent chain extension reaction, residual Dmtr-pyrrole was removed by precipitation of the oligonucleotidyl homostar product into diethyl ether to provide an accurate mass balance, and the purified tris(oligonucleotidyl) homostar was again analyzed by NMR, MS, and HPLC.

It had been hoped that it would be possible to carry out only one CVD per cycle by detritylating immediately after chain extension. However this was not successful, and led to many unidentified side products of longer HPLC retention time if left for any length of time (*Supporting Information*). Accumulation of even low levels of such contaminants with repeated cycles of chain extension could not be risked, which is why we have adopted the double CVD approach reported here.

2.5. Analytical Methods

During the chain extension cycle, all reactions were monitored by high performance liquid chromatography (HPLC). CVD purification was also followed by HPLC to determine when the retentate could be removed from the rig. An *Agilent* 1100 Series HPLC system was employed equipped with a UV detector and *Varian* 385-LC evaporative light scattering detector (ELSD). The pump flow-rate was set at 1 ml.min⁻¹, the injection volume was 30 µL, the column temperature was 30 °C, and an ACE C18 RP column (*Hichrom Ltd, UK*) was fitted. The column was eluted with a gradient of MeOH and water buffered with 100 mM ammonium acetate. The UV wavelength was set at 260 nm, the ELSD evaporation temperature was set to 40 °C, nebulization temperature at 55 °C, and the nitrogen gas flow rate was at 1.5 SLM (standard liter per minute).

Nuclear magnetic resonance (NMR) spectra were recorded on Brüker AV-400 or Brüker AV-500 spectrometers. Mass spectra (MS) were recorded on *Micromass* MALDI micro MX, or *Micromass* LCT Premier (ESI) mass spectrometers.

3. Results & Discussion

3.1. Membrane Screening & Characterization

Several different types of membranes, including polyimide, polyamide thin film composite (TFC), ceramic membranes, and a cross-linked polybenzimidazole (PBI) membrane were screened for use in LPOS-OSN. The criteria for membrane selection were: 1) chemical and solvent stability, 2) high rejection of the growing oligos (three *i*-mers attached to the hub molecule, where $i = 2$ to n), 3) low rejection of the impurities (excess monomers), and 4) durability and longevity. Polyimide and TFC membranes were not stable for prolonged periods under the oligonucleotide synthesis conditions, as shown in the work of Valtcheva *et al.*,²² and thus were discarded. Of the other types of membrane, most did not reject the product highly enough while simultaneously allowing the impurities to permeate through. However, PBI membranes cross-linked with DBX (PBI 17DBX) exhibited promising PEG rejection data, shown in Figure 5. Furthermore, the chemical stability of PBI membranes and their reproducible performance in solvent environments had already been demonstrated in the work of Valtcheva *et al.*²² Please see Supporting Information for full screening data.

PBI 17DBX membranes showed exceptional chemical stability towards all the reagents used in this work, as well as reliable mechanical stability and long service life, with excellent durability. It was determined that the threshold pressure of PBI 17DBX membranes is approximately 15 bar, *i.e.* above 15 bar the membrane underwent irreversible compaction and a permanent loss of membrane performance. Hence, the membrane was operated at 5-10 bar at all times, which ensured constant membrane performance for more than a year (See Supporting Information for long-term data). The membrane permeance remained virtually constant at approximately $8 \text{ L.m}^{-2}.\text{hr}^{-1}.\text{bar}^{-1}$ over the entire duration of the 2'-*O*-methyl RNA phosphorothioate nonamer (9-mer) synthesis. Slight variations in flux were observed, depending on the solution concentration and solvent composition, but periodic testing of the PEG rejection by the membranes between preparative experiments confirmed that the performance remained substantially unchanged.

3.2 Justification for Using a Branched PEG Homostar Support

When performing solute fractionation operations using membranes, the constant volume diafiltration (CVD) mode is usually employed, where the retentate volume is held constant by matching the permeate volume outflow with pure solvent input. In CVD, smaller solutes generally permeate through the membrane faster than bigger solutes: the slower the permeation, the higher the rejection by the membrane, and vice versa (Eq. 2). Consequently, the greater the difference in solute permeation rates (or rejections), the easier the separation becomes. The term rejection is mathematically defined as the following:

$$R_i(\%) = \left[1 - \left(\frac{C_{Pi}}{C_{Ri}} \right) \right] \times 100\% \quad (\text{Eq. 2})$$

where C_{Pi} and C_{Ri} represent the concentrations of species i in the permeate and retentate, respectively.

In this work the growing oligos [supported as tris(oligonucleotidyl) homostars] are the products that must be highly retained by the membrane, whereas it is desirable that everything else (excess building blocks and other reaction debris) should permeate through. By writing a mass balance around the system (see Appendix A), it can be shown that to achieve a clean and efficient separation between large and small solutes using diafiltration, two conditions need to be met: 1) the difference in rejection between the two solutes needs to be wide; and 2) the rejection of the larger compound should be as close to 100% as possible. The first condition determines the efficiency of separation, whereas the latter determines the yield of the process.

This is illustrated in Figure A1 (Appendix) where the normalized solute concentration (C/C_o) profile is plotted against the number of permeated *diavolumes*. It is important to note that even with a seemingly high product rejection of 95%, the remaining concentration (*i.e.* diafiltration yield) after 20 *diavolumes* is only 38%. Therefore, to obtain a high diafiltration yield, the growing supported oligos must have near to 100% rejection.

In the initial work the growing oligo was covalently attached to a long-chain linear PEG support³¹. PEG was selected as a polymer support because of its high solubility,

easy functionalization, and low cost. In addition, different molecular weights (MW) were readily available. The PEG support increased the rejection of the oligo relative to the debris, and widened the rejection difference between them, improving the discrimination and efficiency of separation. However, it quickly became clear that it is difficult to obtain rejections higher than 95%, regardless of the MW of the PEG. It was hypothesized that because of the PEG's highly flexible nature, it could thread, or reptate through the membrane pores, lowering its rejection below that typically expected for more globular molecules of a similar MW.

Figure 5. Comparison of linear *versus* branched PEG rejection by PBI 17DBX membranes. Linear PEG rejections, regardless of their MW, do not exceed 95%. Branched PEG compounds exhibit higher rejections and approach 100% above 3,000 Da.

Because of these initial observations, we investigated the potential of a branched PEG synthesis support, as it has been shown that branched solutes exhibit higher rejection than linear ones of comparable MW³². The use of a branched support has the additional benefit of increasing the loading of oligos per support molecule – from a maximum of only two sites per linear PEG, to one site for every side-arm on a branched support. 1,3,5-Tribromomethyl benzene was treated with a 10-fold excess of linear PEGs, MWs ranging from 200 to 2,000 Da, to give crude mixtures containing approximately

seven parts starting linear PEG mixed plus one part 1,3,5-tris(PEG-oxymethyl)benzene. The rejections of linear and branched, 3-armed PEG homostars by PBI 17DBX were determined and are plotted against MW, Figure 5.

As can clearly be seen in Figure 5, whereas the rejection of the linear PEGs rose to around 95%, but did not rise higher with growing molecular weight, above *ca.* 3,000 Da the rejection of branched PEG homostars approached very closely to 100%. The significance of this apparently small difference should not be understated, as it makes a large difference to the overall yield after many *diavolumes* have permeated, as illustrated in Figure A1. Thus it was anticipated that synthesizing oligos extending out from a central branched synthesis support would much improve the yield over our initial approach based on linear PEG supports.

Taking the homostar rejection effect as a general principle, we then realized that a monodisperse PEG support for oligo synthesis would have significant advantages over a more easily prepared poly-disperse support: its complete monodispersity would ensure a discrete molecular entity, with a single MW (unlike a polydispersed support) facilitating convenient analysis by mass spectrometry (MS), as well as NMR and HPLC. An octagol homostar (1224 Da) was chosen as the monodisperse support³⁰ for this work because once the second residue (cytidine in the present work) is attached, the size of the dinucleotidyl PEG homostar conjugate has already become large enough (2245 Da) to be highly rejected by the PBI 17DBX membrane (>99%). In addition, as the oligo chains grow, the rejection is expected to approach 100%.

3.3 Chain Extension & Detritylation (combining reactions and diafiltrations)

Using the procedure illustrated in Figures 3 and 4, we have successfully synthesized 2'-O-methyl RNA phosphorothioate pentamer (5-mer) and nonamer (9-mer), with the sequences shown in Figure 4. The syntheses of 5-mer and 9-mer protected homostars started from 0.7 g and 1.4 g of mono-nucleosidyl homostar **3**, respectively. However, as the oligos became longer, the reaction scale varied depending on the yield after each step, and how much sample was retained for further analysis.

In this work the couplings employed a relatively low excess - 1.5 equivalents (eq. per OH) – of phosphoramidite monomer **5**. This compares favorably to more than 2 eq. excess monomer typically employed in the reported SPOS strategies¹⁴, although the exact excesses used commercially are not publicly available. It should be stressed that in oligonucleotide synthesis, the main economic driver is the phosphoramidite excess (discussed in Section 3.5).

The post-reaction crude mixture contained multiple nucleotidyl byproducts. These resulted from the conversion of the excess phosphoramidite monomer a mixture of four different phosphoryl derivatives upon addition of PADS to the coupling mixture, as shown in Figure 6. Apart from the monomer excess, the crude mixture also contained ETT and its diisopropylammonium salt, pyridine, and a large number of PADS-related species.

Figure 6. Monomer debris identified during the chain extension cycle. Thioamidate (**6a**, Dmtr or H) and amidate (**6b**, Dmtr or H) are neutral species, whereas monothioate (**6c**, Dmtr or H) and dithioate (**6d**, Dmtr or H) are charged species.

Because LPOS-OSN is a liquid phase method, it is straightforward to sample at any stage of the process and to utilize versatile and highly informative analytical techniques such as HPLC, NMR, and MS. HPLC can be used to determine if the reaction has reached completion or not (See the Supporting Information), and also whether the impurities have been completely removed. Since ³¹P NMR only detects phosphorus-bearing compounds, this tool is able to show what types of monomers were present, and also whether they have been removed or not. MS was mainly used to confirm the identity of the product, and for the dimer and trimer MS was also used to identify some minor

product-related impurities. The HPLC, ^{31}P NMR, and MS data of the dinucleotidyl homostar (tritylated **7**, and detritylated **8**) are presented in Figure 7.

Figure 7. Analysis of a typical chain extension cycle, from mU loaded homostar **3** to 5'-OH dinucleotidyl homostar **8**. Each peak has been identified by comparison with pure compound. **a)** HPLC of feed, retentate and permeate after chain extension, but before detritylation. Small molecule reagents – PADS, ETT, and pyridine – permeate through the PBI membrane, but monomer-related species are retained. **b)** HPLC after detritylation; excess monomers permeate but Dmtr-pyrrole only permeates partially. **c)** ^{31}P NMR of retentates before and after detritylation. Three classes of monomer debris (except thioamide **6a**) are present before detritylation, but are absent after detritylation. **d)** MALDI-TOF+ MS confirming the presence of compound **7** and **8**. Also, *N*-

deacetylation of cytosine base for **8**, a common impurity, is identified ($[\mathbf{8}\text{-Ac}+2\text{H}]^+ = 3495.6$, calc. $\text{C}_{142}\text{H}_{212}\text{N}_{18}\text{O}_{71}\text{P}_3\text{S}_3^+ = 3495.2$).

Figure 7 summarizes a typical set of data obtained for the first chain extension cycle; although the HPLC chromatogram becomes broader for longer oligos (discussed below), the ^{31}P NMR and MS data are also representative of later cycles. Figure 7a depicts the HPLC traces of dinucleotidyl homostar **7** (5'-*O*-Dmtr) post-chain extension, both before and after membrane CVD. All the HPLC peaks were resolved except for the thioamidate (**6a**, R=Dmtr) peak, which overlapped with the product **7** (5'-*O*-Dmtr). PADS showed more than one HPLC peak and their relative intensities varied with the batch. Figure 7a demonstrates that all the small reaction debris, *i.e.* ETT, pyridine, and PADS, were completely removed in the first diafiltration. However, all four types of excess monomer debris (**6**, R=Dmtr) remained in the final retentate, as they were all highly rejected (>95%) by the PBI membranes. The main reason for such high rejections was assumed to be the bulky 5'-*O*-Dmtr protecting group.

Figure 7b shows HPLC traces for the same experiment, but post-detritylation of dinucleotidyl homostar **8** (5'-OH), where the thioamidate (**6a**, R=H) and amidate (**6b**, R=H) peaks overlapped with the product **8**. After membrane CVD, only two peaks were present in the final retentate: dinucleotidyl homostar **8** (5'-OH), and Dmtr-pyrrole. Complementing the HPLC data, it can be seen in the ^{31}P NMR (Figure 7c) that after detritylation all the charged thioate monomers (**6c**, **6d**, R = H) were removed efficiently by diafiltration. However, thioamidate (**6a**, R=H) and small traces of amidate (**6b**, R=H) monomers were detected in the final retentate (thioamidate **6a** not shown in Figure 7, explained below). The measured rejection of amidate (**6b**, R=H) was ~85, but the rejection of thioamidate (**6a**, R=H) was significantly higher at >99%. Thus, the amidate (**6b**, R=H) was mostly removed by the second diafiltration, but the thioamidate (**6a**, R=H) remained as a major component of the monomer debris.

Notably, the ratios of the concentrations of the four types of monomers varied considerably from one chain extension to the next. Since the relative amounts of monomer debris were only quantified (by ^{31}P NMR) after the first CVD, this variation may be partially explained by slight differences in the rejections of the monomers.

However, it was found that reducing the excess of PADS from 10 to 3 equivalents per 5'-hydroxyl consistently reduced the proportion of thioamidate generated to undetectable levels. Thus, it was beneficial to use the lower excess of PADS to minimize the amount of thioamidate generated, and so to maximize oligonucleotidyl homostar purity at the end of each chain extension cycle. Figure 7d presents the MALDI MS data for the tritylated and detritylated dinucleotidyl homostar (**7** and **8**, respectively), which clearly shows the expected peaks of $[7+\text{Na}+\text{H}_2\text{O}]^+ = 4484.1$ and $[8+\text{H}]^+ = 3538.3$.

The only significant contaminant remaining at this point, after the second CVD, was Dmtr-pyrrole. Although this molecule does permeate PBI 17DBX (see permeate in lower trace, Figure 7b), its absolute quantity and rejection was too high (>90%) to be removed completely. Despite the relatively low MW of Dmtr-pyrrole, it was assumed that its bulky shape, as well as a possible contribution from its hydrophobicity, made it difficult for this molecule to permeate through the membrane. Although it probably does not interfere with the next chain extension, in order to obtain accurate mass data we deliberately precipitated the growing oligonucleotidyl homostar in diethyl ether to remove the remaining Dmtr-pyrrole. Undoubtedly, this step should be avoided or replaced in a scalable LPOS strategy, either by using a different membrane, or a different protecting group.

3.2.2 Ion Exchange Transport through PBI membranes

After detritylation, it was initially observed that the rejection of thioate monomers (**6c**, **6d**, R=H) was over 95% in MeCN, making CVD impractical. Surprisingly, upon addition of 1 vol% DCA, the thioate monomers began to permeate. Unfortunately, the high concentration of DCA caused significant capping of the 5'-OH in the crude tris(HO-dinucleotidyl) homostar **8** as dichloroacetate esters, as well as acid catalyzed *N*-deacetylation of the protected cytosine nucleobase. Assuming that thioate permeation proceeded via an anion exchange mechanism, the DCA was later replaced by 1 vol% pyridinium dichloroacetate (Py.DCA in 20% MeOH-MeCN), when it was again found that the anionic thioate monomers (**6c** and **6d**, R=H) permeated with rejections lower than 50%, but *N*-deacetylation was now very low. Taking into account that the thioate monomers did not permeate at the tritylated stage (**6c** and **6d**, R = Dmtr), this suggests

that their permeation occurs through a combination of both ion exchange and sieving mechanisms. However, the higher rejections of neutral monomers (**6a** and **6b**, R=H) were unaffected. Considering that the difference in MW between the anionic thioate monomers and neutral monomers is less than 84 Da, the differences in rejections were quite remarkable.

From this behavior it was hypothesized that the anionic thioate monomers (**6c**, **6d**), paired with diisopropylammonium cations, permeated via an anion exchange mechanism. Such a mechanism would be consistent with the poly-basic structure of cross-linked PBI²⁹, which can deprotonate Py.DCA ($\text{PyH}^+ \cdot \text{Cl}_2\text{CHCO}_2^-$ ion pair). In addition, the PBI backbone may contain some permanent positive charges on cationic imidazolium rings that form during the membrane cross-linking step, shown in Figure 8. The addition of Py.DCA could facilitate the movement of membrane-associated thioates (**6a**, **6b**, R=H) through the membrane by exchange of anions between the immobile imidazolium cations and pyridinium cations in solution. A speculative transport mechanism is illustrated in Figure 8.

Figure 8. Speculative transport of anionic thioate monomers (**6a**, **6b**, R=H) through PBI membranes via the ion-exchange mechanism. The cationic imidazole backbone allows

anionic species to exchange between the sites, pushing out the thioates to the permeate side down the concentration gradient.

3.2.3. Synthesis of 9-mer

Figure 9. HPLC chromatograms up to 7-mer oligos (from 9-mer preparation). Tritylated (-ODmtr) compounds have longer retention times than detritylated (-OH) species. The peaks became too broad to be detected by HPLC after 7-mer.

From the outset, the choice of a mono-disperse support was seen as an opportunity to exploit HPLC throughout oligo synthesis. Usefully, tritylated (-ODmtr) oligo-homostars elute from the HPLC column later than detritylated (-OH) species, owing to the hydrophobicity of the Dmtr protection. However, the peaks gradually broadened with longer chain length, so that by 6-mer it was virtually impossible to detect and assay the oligonucleotidyl homostar by HPLC (Figure 9). One of the reasons for this peak broadening is thought to be the exponential increase in the number of diastereoisomers in the oligonucleotidyl homostar's phosphate backbone. For instance, the number of diastereoisomers quadruples from 2-mer (four) to 3-mer (sixteen) (See the Supporting Information). Furthermore, the solubility of the oligos in the HPLC mobile phase

(MeOH-water) decreased with the chain length, necessitating the use of more dilute sample leading to weaker peak intensity. Hence, from 7-mer **17** onward we mainly relied on the ^{31}P NMR for assessing the oligo purity. Nevertheless, HPLC has proved to be an effective analytical tool to monitor the extension reaction, as well as to detect the presence of impurities. In order to analyze the oligonucleotidyl homostars beyond 7-mer, if no effective HPLC method can be found to monitor the intact oligonucleotidyl homostars, a destructive method to remove the diastereoisomeric artifact (*e.g.* global deprotection) could be employed.

3.2.3. Solubility of the Growing Oligonucleotidyl Homostar

The oligos' solubility changed drastically with the growing chain length. It was observed that their solubility in neat MeCN rapidly decreased with chain length, so that while 2-mer **8** had moderate solubility in MeCN, 4-mer **12** was almost insoluble. However, upon the addition of MeOH to the solution, the oligo solubility was markedly enhanced (5-20% MeOH in MeCN). Notably, the oligos were insoluble in both neat solvents, dissolving only in the mixture. The same was true with the chlorinated solvents, where the growing oligos could be dissolved in mixtures of chloroform or dichloromethane with methanol, but in neither alone. This suggests these species require a protic solvent, possibly to disrupt hydrogen bonding of the nucleobases, to be solvated. In addition, a higher fraction of MeOH was required with the increasing chain length, possibly due to greater opportunities for intermolecular H-bonding with larger molecules. Even so, the 9-mer **22** had excellent solubility (above 10g.L^{-1}) in 20% MeOH-MeCN.

Apart from the MeOH-MeCN mixtures, the oligonucleotidyl homostars also exhibited excellent solubility in neat DMF (above 100g.L^{-1}) and DMF-MeCN mixtures. Even though oligonucleotide phosphoramidite couplings have been reported in DMF¹⁹, we noticed that the chain extension reaction was not as fast in neat DMF as in MeCN. As the chemistry of the growing oligos is quite complex, it seems inevitable that a mixed solvent will be required for both synthesis and purification. Nevertheless, a single-solvent system for both reaction and separation is highly desirable from the process perspective and will be explored further.

3.2.4. Yield of Oligonucleotide Synthesized by LPOS

Chain extension yields and the corresponding overall yield for 9-mer synthesis are summarized in Figure 10 below. Yield values were calculated from the measured mass data assuming 100% product purity, and are reported as molar yield.

Figure 10. Summary of chain extension yield and the corresponding overall yield, starting from the mononucleosidyl homostar **3** as the basis of calculation: The yield is calculated from the mass data and reported as molar yield, assuming 100% purity.

It was anticipated that bigger oligonucleotidyl homostars would exhibit higher rejections by the membrane. In Figure 10 it can be seen that the step-wise yield improved with the chain length as expected. Indeed, from the 5-mer onward the average step-wise yield was higher than 95%. It can also be seen that the major loss in the overall yield resulted from the first three chain extension cycles, contributing half of the total loss. Although the rejection of the oligonucleotidyl homostar at the dimer stage was already high at 99%, the isolated yield after 25 *diavolumes* was only 75%, as predicted from Figure A1 (Appendix). The rejection of the oligonucleotidyl homostars rose to 99.8% after tetramer. However, it should be noted that the calculated yield was solely based on the mass data and the purity was not taken into account (only determined after global deprotection). Hence, the apparent observed yield measurement must be higher than the

actual yield of pure oligo. Nevertheless, the rejection of the product is approximately correct based on the HPLC integration, and the obtained yield values are likely to be within errors.

It is often difficult to obtain a complete rejection (100%) using membranes, and rejection values between 95 – 99.9% are commonly observed even for very tight membranes³³. This is due to a low level of defects that inevitably arise during the fabrication process, and/or to the permeation mechanism through the membranes. Generally, the defect-related drop in rejection is eliminated when membrane modules are employed, due to the much larger area to system volume ratio. Nevertheless, it is anticipated that the maximum yield per chain extension is likely to be below 100% due to incomplete rejection. To compensate for the significant losses in the first three chain extension cycles, an alternative membrane configuration could be applied, such as a membrane cascade^{34, 35}, which has been shown to increase the process yield without compromising the purity.

3.4. Final deprotection and Purity Analysis

Figure 11. Final purity analysis by HPLC after global deprotection: **a)** 5-mer oligo prepared by LPOS-OSN using 1vol% DCA; and **b)** 5-mer oligo by LPOS-OSN using 1vol% Py.DCA; **c)** 9-mer by LPOS-OSN, purified by ion exchange (49% before purification); **d)** 9-mer by SPOS after desalting. Purity is roughly estimated by integrating the product peak.

Two different batches of pentanucleotidyl homostar **14**, and one batch of 9-mer homostar **22**, were cleaved from the core octagol-homostar support and analyzed for purity by HPLC (Figure 11). For comparison, a 9-mer oligo sample of the same sequence was also synthesized using a typical automated SPOS protocol. The main peaks are indeed the desired 5-mer (**23**) and 9-mer (**24**) oligos; the purity was assessed by comparing the integral ratio of the product peak to the other minor peaks. It can be seen that the 5-mer purity was improved by switching from using DCA during OSN of the detritylated oligo-homostars (66%) to Py.DCA (75%), presumably because capping of the 5'-OH was prevented, consequently reducing the amount of short-mer contaminants

($n-1$, $n-2$, ...and/or $n-i$) visible at shorter retention times. The peaks after the product suggest the presence of long-mer ($n+i$) impurities, which may be generated during chain extension reaction when a newly coupled Dmtr-nucleotide was detritylated by the mildly acidic ETT activator, leading to double chain extension. For this study we had employed long coupling reaction times (>30 min) to allow time for concurrent HPLC analysis. It is likely that many of these longer retention time contaminants can be largely eliminated by reducing the coupling times to a more typical 3-6 minutes.

In general, the purities of oligos prepared by LPOS were not as high as those prepared via SPOS. Compared to the impurity profile of SPOS derived oligos (Figure 11d) where short-mers and long-mers peaks are clearly identifiable, the impurity profile of the LPOS-prepared oligos (Figure 11a-c) showed many irregular and unidentified peaks that do not overlap with the SPOS contaminants. Thus, a simple HPLC purity comparison may not be precise. In the future, LC-MS techniques will be employed to understand this impurity profile.

Nevertheless, the final purity of 9-mer was lower than anticipated (49% before chromatographic purification), mainly because the last coupling reaction had not reached completion (17% of $n-1$ impurity observed, confirmed by LC-MS). It is possible that the chain extension did not reach completion because the 5'-mGmG dinucleotide is the most hindered inter-nucleotide linkage to form. Even so, it can be seen that upon purification of the 9-mer using ion exchange chromatography, the purity can be increased up to acceptable standards (> 90%)⁶.

3.5. Economic Analysis

Currently, the major impediments for SPOS scale-up are the associated costs, and the need for larger SPOS synthesizers. For instance, the cost of a 1kg scale synthesizer is approximately £2 million. Also, the price of oligo therapeutics prepared by SPOS varies significantly depending on the synthetic routes and chemical modifications. On the other hand, the chief advantage of membrane-based processes is that they can be implemented on almost any scale. To explore the competitiveness of the LPOS-OSN platform, an

economic evaluation of the proposed process was carried out from the data obtained in this work.

The basis for calculation was the synthesis of 1 kg of 23-mer oligoribonucleotide. The required reagents and solvents were back-calculated accordingly. Some necessary assumptions were made: (i) initial capital investment on the membrane rig of £250,000 was amortized with a capital charge factor of 0.2; (ii) a total of 20 batches per year was assumed (based on the operation time and membrane permeability of $8 \text{ L.m}^{-2}.\text{hr}^{-1}.\text{bar}^{-1}$); (iii) the system volume was fixed at 100L (based on the required concentration); (iv) only acetonitrile was used as the solvent; and (v) the labor and operation costs were not taken into account. Although the membrane process is considered a low labor-intensity process³⁶, an exact operation cost was difficult to estimate at this stage. It was found that solvent accounts for a large proportion of the overall cost, and hence two different scenarios were considered: the case with solvent disposal, and a scenario with 90% solvent recovery. The calculated cost of solvent recovery (using steam for distillation) contributes to 4% of the overall solvent cost. The overall cost breakdown is shown in Table 1 and Figure 12 below.

Table 1. Overall cost breakdown per 1kg batch of oligo

Item	Calculations	Solvent Disposal	Solvent Recovery
Capital Cost	Amortize by 0.2, 20 batches.yr ⁻¹	£2,500	£2,500
Phosphoramidite Monomer	1.5 eq., £10.2.g ⁻¹	£53,000	£53,000
PADS, ETT, DCA, etc		£1,500	£1,500
Solvent	Acetonitrile, £0.8.L ⁻¹	£60,700	£6,300
Membrane	24m ² , £1600.m ⁻² 10 batches lifetime	£3,800	£3,800
Homostar support		£100	£100
TOTAL (kg⁻¹)		£121,600	£67,200
TOTAL (g⁻¹)		£122	£67

Figure 12. Overall breakdown of the cost: (left) with solvent disposal and (right) with 90% solvent recovery. The two largest fractions are the solvent and phosphoramidite monomer costs. Implementing a distillation solvent recovery unit significantly reduces the overall cost.

For the purpose of the calculation, the overall yield of 23-mer was assumed to be 50% (step-wise yield of 97%). In the first case of solvent disposal, it is quite clear from Table 1 and Figure 12 that the two main cost components are the phosphoramidite monomers and the solvent; other parameters, such as capital cost and membrane modules, are relatively insignificant. The cost of phosphoramidite monomers has come down significantly in the past 10 years in the face of rising demand^{37, 38}, but it is still a major obstacle for commercial oligo production. In addition, the cost of phosphoramidite depends significantly on the scale.

However, it can be seen that the overall cost of solvent when it is not recovered is even higher than the phosphoramidite monomer itself. This is not surprising considering that membrane CVD is a highly solvent-intensive process³⁹ and acetonitrile is one of the most expensive common solvents. For this reason, several approaches have been reported that couple solvent recovery to membrane CVD^{35, 36, 40}. For the purpose of this calculation, we have assumed 90% solvent recovery via distillation and have implemented the associated energy cost. Distillation was chosen as a suitable solvent recovery unit as the only other significant volatile compound employed in this project was pyridine. If 90% solvent recovery is now taken into account, the cost of the oligo drops by 50% and the biggest fraction of cost then becomes the phosphoramidite monomers.

It should be stressed that the labor and operation costs have not been taken into account. Nevertheless, it can be asserted that the proposed LPOS-OSN platform is not only highly competitive with the SPOS platform, but also more easily scalable. To understand which operation parameters affect the overall economics, different sensitivity analyses were carried out, as shown in Figure 13.

Figure 13. Sensitivity analyses aiming to understand how changes in key parameters affect the overall cost: **a)** effect of monomer excess; **b)** effect of overall yield; **c)** effect of membrane area; and **d)** effect of monomer cost.

Several trends can be observed in Figure 13. Firstly, for all cases the recovery of solvent is economically beneficial, and environmentally desirable. Secondly, Figure 13a demonstrates that there is a linear relationship between the molar excess of monomer and the final oligo cost. For instance, decreasing the monomer excess from 1.5 to 1.1 eq. can reduce the final product cost by up to 21%, something that is possible for an LPOS platform where the reaction proceed in a homogeneous liquid phase, and mass transfer does not limit reaction rates, thus allowing a lower reagent excess. Thirdly, the overall yield has a steep inverse exponential relationship with the final cost (Figure 13b), and a steeper gradient for the solvent-recovery case. For instance, increasing the overall yield

from 50% to 100% decreases the required monomer amount by 42%, and decreases the overall cost by 27%.

Beyond these major considerations, it is very important to optimize the total operation time with respect to the membrane area (Figure 13c). Because the membrane area can easily be increased by using additional membrane modules, and the operation time drops with higher membrane area, the operation time is an independent variable under control. It should be stressed that the cost of membranes is only a small fraction (3-6%) of the overall breakdown of the cost (Figure 12). It is expected that the membrane area will be chosen based on the required campaign schedule considering the number of batches, reaction time, and available reactor schedule, etc. The main cost optimization parameters would be the overall yield and the monomer excess. Lastly, as expected, the price depends significantly on the monomer price. If the monomer cost can be reduced down from £10.2.g⁻¹ to £3.5.g⁻¹, with increasing batch scale, the final product cost drops by 52%.

3.6. Further Discussions: advantages and current challenges

The two batches of oligos synthesized here using the LPOS-OSN process clearly demonstrated the expected advantages, such as efficient reaction, minimal usage of phosphoramidite monomers, simple purification methodology, easy scalability, and *in situ* analysis. However, several challenges have been identified during the development stage, and the process needs to go through continuous improvement and optimization. The current challenges are: 1) incomplete removal of excess monomers, especially the amidate **6b**, leading to lower purity; 2) low process yield; 3) incomplete removal of Dmtr-pyrrole requiring a separate precipitation step; and 4) redundant solvent exchange steps.

As for the first challenge, the excess phosphoramidite monomers are converted to four different types of debris upon addition of PADS (Figure 6). By understanding the mechanism of their formation, the reaction should be pushed towards the thioates (**6c**, **6d**), which have been shown to permeate more easily than amidates (**6a**, **6b**) through the PBI membranes, probably via an ion exchange mechanism. The second challenge, low

process yield, can be tackled through different membrane configurations. For example, recent work by our group showed that employing a two-stage cascade can increase the yield without compromising the product purity^{34, 35}. In addition, OSN membranes have also shown potential for an in-situ solvent recovery³⁹. Also, further improvements in yield and purity can be made by parallel tuning of the size of the soluble support and the membrane separation performance, to make the purification more efficient. Notably, monodisperse PEG homostars have been prepared up to 8,000 Da^{30, 41}, and the membrane separation performance can be roughly controlled using polymer fraction in the dope and the solvent to co-solvent ratio⁴². It should be noted that even though a bigger soluble support may exhibit higher rejection, it also decreases the oligo loading (mmols.g⁻¹ of PEG support) while increasing the solution viscosity.

The use of Dmtr protecting group has been extremely versatile and useful for the SPOS process, but it has proven to be too bulky for the proposed LPOS-OSN process, at least with the PBI 17DBX membrane, requiring a precipitation step after every chain extension cycle. Instead, other acid-labile protecting groups should be explored. For example, the methoxy isopropyl acetal protecting group (MW 74) employed by Molina *et al.*^{43, 44} to synthesize 3-mer oligos on a cyclodextran support could be applied.

Lastly, the current chain extension protocol requires redundant solvent exchanges and a precipitation step, rendering the practical operation of the process cumbersome and time-consuming. From the process operation perspective, a single-solvent and single-pot synthesis protocol is preferred. One way to achieve significant process intensification is to bypass the detritylation reaction in DCM, and perform the deprotection reaction and membrane CVD in the rig (the membrane reactor concept). This is expected to significantly simplify the process, as the entire chain extension cycle can proceed with effectively a single CVD using a single-solvent system. All of these challenges are currently under investigation and will be covered in our future publications.

Apart from the practical challenges identified in this work, the LPOS-OSN concept still needs to prove its versatility and that it can produce other species apart from antisense oligos, for instance spiegelmers, and locked nucleic acid (LNA), among other types of oligo-based drugs.

4. Conclusions

In this work we have introduced a new liquid-phase oligonucleotide synthesis (LPOS) platform using organic solvent nanofiltration (OSN) as a scalable purification technology. By growing oligos on a soluble, branched monodispersed PEG support, purification could be performed after each reaction using OSN membranes, exploiting the difference in size between the product oligonucleotidyl homostar and smaller reagent debris. Using this new platform, 5-mer and 9-mer 2'-*O*-methoxy phosphorothioate oligoribonucleotides have been successfully synthesized and characterized. The solubility of the oligonucleotidyl homostars was excellent in mixed MeOH-MeCN solvent, as well as in DMF. The synthesis was carried out on a gram-scale, which could easily be scaled-up to kilo-scale using membrane modules. The main advantage of the proposed process is that its performance is not affected by the scale of synthesis. We have chosen a PBI membrane as it gave reliable and robust performance for over a year. A thorough economic analysis was carried out for the LPOS-OSN platform to understand the main cost factors, as well as operation parameters.

Although this work has shown the potential of the new liquid-phase platform for oligo synthesis, continuous improvements and optimization steps must be taken. Several process challenges have been identified, and possible solutions suggested. The proposed LPOS-OSN platform presents a flexible alternative for the large-scale synthesis of oligos, and allows economical synthesis of oligo-based therapeutics on a metric-ton scale. With the ever-growing number of oligo-based drugs going through clinical trials, a scalable manufacturing technology is expected to further boost growth in this exciting new era of oligo therapeutics.

List of Abbreviations

Term	Full name	Term	Full name
Cne	cyanoethyl protecting group	MeCN	acetonitrile
CVD	Constant Volume Diafiltration	MeOH	methanol

Da	Daltons [g.mol ⁻¹]	MS	Mass Spectrometry
DBX	dibromo- <i>p</i> -xylene	MW	Molecular weight
DCA	dichloroacetic acid	NMI	<i>N</i> -methylimidazole
DcbCl	2,6-dichlorobenzoyl chloride	NMP	<i>N</i> -methyl-2-pyrrolidone
DMAc	<i>N,N</i> -dimethylacetamide	NMR	Nuclear magnetic resonance
DMF	<i>N,N</i> -dimethylformamide	N ⁿ	<i>n</i> th nucleotide
Dmtr	4,4'-dimethoxytriphenylmethyl	OSN	Organic Solvent Nanofiltration
DNA	deoxyribonucleic acid	P	Protecting group
ELSD	Evaporative light scattering detector	PADS	phenylacetyl disulfide
ETT	S-ethylthiotetrazole	PBI	polybenzimidazole
FDA	Food and Drug Administration	PEG	polyethylene glycol
HPLC	High pressure liquid chromatography	Py.DCA	pyridinium dichloroacetate
IPA	isopropyl alcohol	RNA	ribonucleic acid
<i>J</i>	flux [L.m ⁻² .hr ⁻¹]	RNAi	RNA interference
LNA	locked nucleic acid	SPOS	Solid-phase Oligo Synthesis
LPOS	Liquid-phase oligo synthesis	TFC	thin film composite
		TLC	thin layer chromatography

5. Appendix A: Process Modeling

A constant volume diafiltration (CVD) system can be modeled by writing a mass balance around the system. Assuming that the system operates at a constant volume and it is perfectly mixed,

$$V \frac{dC_{R,i}}{dt} = -F \cdot C_{p,i} = J_v \cdot A \cdot C_{p,i} \quad (\text{Eq. A1})$$

where V (dm³) is the entire system volume, F is the permeate flow-rate (dm³.hr⁻¹), J_v (dm³.m⁻².hr⁻¹) is the membrane flux, A (m²) is the membrane area, and $C_{R,i}$ and $C_{p,i}$ (g.dm⁻³) are the concentrations of species i in the retentate and permeate, respectively.

Defining the observed rejection of species i as,

$$R_{obs} = 1 - \frac{C_{p,i}}{C_{R,i}} \quad (\text{Eq. A2})$$

substituting Eq. (A2) into Eq. (A1) yields,

$$\frac{dC_{R,i}}{dt} = -\left(\frac{1}{V}\right) J_v A C_{R,i} (1 - R_{obs}) \quad (\text{Eq. A3})$$

This equation can be solved either numerically or analytically. When integrated analytically with appropriate boundary conditions, the following equation is obtained:

$$\frac{C_{r,i}}{C_{r,i,o}} = \exp\left[-\frac{J_v A t}{V} (1 - R_{obs})\right] = \exp[-diavolume (1 - R_{obs})] \quad (\text{Eq. A4})$$

where *diavolume* represent the total volume of permeate collected relative to the initial system volume. This useful *time-like* dimensionless parameter allows different diafiltration systems to be compared.

Figure A1. The effect of rejection on the overall concentration profile (*i.e.* diafiltration yield versus *diavolumes* at different solute rejections).

6. Acknowledgement

The authors wish to acknowledge the following supports: J.F.K. and I.B.V. thank the 7th framework program of the European commission's Marie Curie initiative PITN-GA-2008-238291-MEMENTIDE; P.R.J.G. thanks the EPSRC for a Platform grant EP/J014974/1 and GSK.

7. Description of the Supporting Information

The supporting information file for this work includes: (1) effect of adding acid to crude reaction, without initial diafiltration; (2) HPLC peak broadening effect with oligo length; (3) effect of membrane cascade on overall process yield; (4) detection of incomplete reaction using HPLC; (5) detailed preparation and screening data for tested membranes; (6) long term performance data of PBI membranes.

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