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Purification of a GalNAc-cluster-conjugated oligonucleotide by reversed-phase twin-column continuous chromatography

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ABSTRACT

Multicolumn Countercurrent Solvent Gradient Purification (MCSGP) is a continuous chromatography technique used to maximize purification yields compared to traditional batch purification methods. Here we apply MCSGP for the reversed phase purification of a *N*-acetylgalactosamine (GalNAc)-cluster-conjugated DNA-LNA gapmer oligonucleotide therapeutic using a twin-column chromatography system. Based on a batch process as a starting point, MCSGP was designed, optimized and compared with the batch process regarding process performance and scale-up requirements. Product yields increased from 52.7% using batch chromatography to 91.5% using MCSGP, with purity, productivity, and buffer consumption otherwise comparable. In a manufacturing scenario, use of MCSGP would allow the downscaling of oligonucleotide synthesis by 42.5%, which would result in a significant cost reduction and increased throughput. Moreover, the equipment, chemicals and methodology used in MCSGP are analogous to a standard reversed phase purification allowing for a "like for like" transition to the upgraded MCSGP process.

1. Introduction

1.1. Chromatographic purification of oligonucleotides

Oligonucleotide compounds are emerging as a major category of therapeutic drugs, due to their ability to regulate gene expression with great specificity. Native oligonucleotide sequences are naturally vulnerable to degradation and clearance by the immune system, and they also fail to permeate through cell membranes to the site of biological action. Recent advances have led to the development of synthetic oligonucleotides modified with nucleic acid analogues that greatly enhance stability and avoid clearance by the immune system [1]. In addition, conjugation with *N*-acetylgalactosamine (GalNAc) ligands provides a mechanism for transport of synthetic oligonucleotides into hepatocytes via endocytosis allowing for the development of treatments that target diseases of the liver [2].

Based on these recent advances in the pharmacological properties of oligonucleotide therapeutics, there is now an increasing pipeline of oligonucleotide drugs in clinical trials and demand for production capacity is rapidly expanding [3]. However, oligonucleotide therapeutics are yet to be manufactured at multi-ton scale and no compounddedicated production facilities exist. Given that oligonucleotide production at larger scale is on the horizon, pharmaceutical companies and CMOs are actively evaluating platform technologies that will optimize production and reduce costs. Oligonucleotide production is done in four major steps, solid phase oligonucleotide synthesis, chromatographic purification, desalting by diafiltration or precipitation and finally isolation by freeze drying [4]. Currently, oligonucleotide synthesis is the major cost driver in production of therapeutic oligonucleotides and any loss of product in the downstream steps has a major impact on the overall manufacturing costs. In particular, the preparative chromatographic purification is a critical step where product losses can be substantial and should therefore be thoroughly optimized.

The goal of the chromatographic purification step is to achieve a clinical grade product purity specification of >90%, which is typically associated with a yield loss between 20% and 50%, and expensive re-chromatography steps to recover product from the side-fractions. Given the diminishing returns of re-chromatography, the upstream oligonucleotide synthesis needs to be scaled up to compensate for the overall yield loss during the chromatographic purification.

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The chromatographic purification of oligonucleotides is challenging, because the synthesis delivers about 60% to 80% desired product together with a mixture of impurities such as shortmers (e.g. N-x), derived from incomplete chemical coupling reactions, full-length product impurities (e.g. cyanoethyl modification, phosphate impurity, depurination), derived from modifying side reactions, and longmers (e.g., lambdamers, branchmers), derived from the incorporation of impurities found in the starting raw materials [5]. Even with considerable optimization efforts, a preparative batch method gives a chromatogram with an elution profile in which impurities are significantly co-eluting with the product, especially for highly phosphorothiolated sequences. Impurities that are chemically very similar to the desired product tend to co-elute. N-1 impurities missing one nucleotide, and $N + 1$ impurities with one additional nucleotide are examples of impurities that are particularly challenging to remove. To meet the product specification, it is therefore essential to carefully fractionate the pure material from the elution peak. Side fractions with lower purity are either discarded or undergo re-chromatography at great cost (lower productivity, increased buffer consumption, intermediate sample storage capacity, additional quality control steps, etc.).

As with any conventional batch chromatographic methods, there is an inverse relationship between product purity and yield depending on separation performance, how the elution peak is fractionated, and product is pooled (Fig. 1A). Overcoming the yield / purity tradeoff can be achieved using recycling chromatography or more advanced twin column-based process technology, such as MCSGP. MCSGP alleviates the yield / purity trade-off by internally recycling the impure product side-fractions to a second column ensuring that only the product reaching the purity specification leaves the system (Fig. 1B). The recycling in the MCSGP process and in single-column based recycling processes such as Steady-state recycling (SSR) and Closed-loop recycling (CLR) is fundamentally different [6–8]. In the latter two processes the chromatographic profile passes through a pump every cycle which is possibly detrimental to product stability and limits the processes to isocratic operation. In MCSGP, impure side fractions that are recycled from one column to the other are fully adsorbed on the second column and then subject to renewed gradient elution to restart the separation. Product and impurities only pass through a pump during the load step, which is beneficial to product stability. This makes MCSGP well suited for the separation of complex molecules such as oligonucleotides. While originally developed as a multicolumn process with 3–6 columns [9,10], over the years MCSGP was simplified to a twin column process, greatly increasing operational flexibility, and reducing complexity. The experimental design of twin column MCSGP is described in detail in [11].

1.2. The MCSGP process principle

MCSGP can be systematically designed with the aid of software by dividing a batch gradient chromatogram into four phases (P1-P4) making up one MCSGP "switch". A step-by-step depiction of the MCSGP process principle is outlined in Fig. 1C. The table shows process steps occurring simultaneously for "column 1" and "column 2" during an MCSGP switch and this is aligned with an example UV trace for column 1, which undergoes product elution during the switch. The table shows the initial state of both columns at $t = 0$ and the four MCSGP phases:

- Initial state – Column 1 is fully loaded during a "startup" method run before the switch or from the previous switch in the MCSGP run; Column 2 requires cleaning and regeneration to remove strongly adsorbing impurities (S) carried over from a prior switch or at the very start of an MCSGP run column 2 would be clean.
- P1 (Columns in parallel) - elution of weakly adsorbing impurities (W) from column 1 to waste; simultaneous regeneration of column 2 ready for loading steps in P2, P3 and P4.
- P2 (Columns interconnected for side fraction recycling) – elution of weakly adsorbing impurities + product (W/P) from column 1, recycled with in-line dilution directly to column 2
- P3 (Columns in parallel) – elution and collection of pure product (P) from column 1; simultaneous loading of new feed on column 2. To maintain a switch-to-switch steady state, the quantity of new feed applied is in equilibrium with product (P) removed. The load required to achieve this is calculated with the aid of software, based on the batch chromatogram and fraction analysis.
- P4 - (Columns interconnected for side fraction recycling) elution of product + strongly adsorbing impurities (P/S) from column 1, recycled with in-line dilution directly to column 2

At the conclusion of switch 1, both columns are immediately ready for switch 2. Column 2 is now fully loaded and column 1 requires cleaning and regeneration. **P1** – **P4** are repeated as described above, but with column positions interchanged (Fig. 1C - yellow arrows). By alternating the position of column 1 and column 2 switch-to-switch, MCSGP can operate continuously. Finally, 1 cycle of MCSGP is defined as 2 switches that includes 1 product elution from each column.

MCSGP has been successfully applied to the purification of peptides, proteins, and DNA-based oligonucleotides [12–15]. Here we evaluate for the first time the application of MCSGP for the purification of a GalNAc-cluster-conjugated DNA-LNA gapmer oligonucleotide using reversed phase chromatography. We demonstrate a major improvement in yield by substituting single column batch chromatography for MCSGP and provide process performance predictions for production at industrial scale.

2. Methods

2.1. Oligonucleotide synthesis and crude preparation

The oligonucleotide KGN.Lo.dCo.dAo.lTs.lEs.lAs.dAs.dCs.dTs.dTs.dTs.dCs.dAs.dCs.dTs.dTs.lEs.lAs.lG (KGN = Bislysin GalNAc cluster; Sequence description: first letter: d = DNA, l = LNA; second letter: A = adenine, C = cytosine, E = 5-methylcytosine, G = guanine, L = linker, T = thymine; third letter: o = phosphate, s = thiophosphate) was synthesized using standard phosphoramidite chemistry on solid phase followed by ultrafiltration and solution phase conjugation to the GalNAc cluster. The solid phase synthesis was performed at a scale of 2.0 mmol using an ÄKTA Oligopilot 100 and Primer Support Unylinker (NittoPhase LH Unylinker 400). In general, 1.5 equivalents of the phosphoramidites (0.2 M in acetonitrile or acetonitrile/dichloromethane = 1/1) were employed. All reagents were used as received from commercially available sources and reagent solutions at the appropriate concentration were prepared:

- activator: 1.0 M 4,5-dicyanoimidazole, 0.1 M *N*-methylimidazole in acetonitrile
- thiolation: 0.1 M xanthan hydride in acetonitrile/pyridine = 1/1 v/v
- oxidation: 0.05 M I_2 in pyridine/water = 9/1 v/v
- detritylation: 10 vol% 2,2-dichloroacetic acid in toluene
- capping A: 10 vol% *N*-methylimidazole, 10 vol% 2,6-lutidine in acetonitrile
- capping B: 20 vol% acetic anhydride in acetonitrile
- backbone deprotection: 20 vol% diethylamine in acetonitrile

Cleavage and deprotection was achieved using 30 wt% aqueous ammonium hydroxide/ethanol = 3/1 v/v for 9 h at

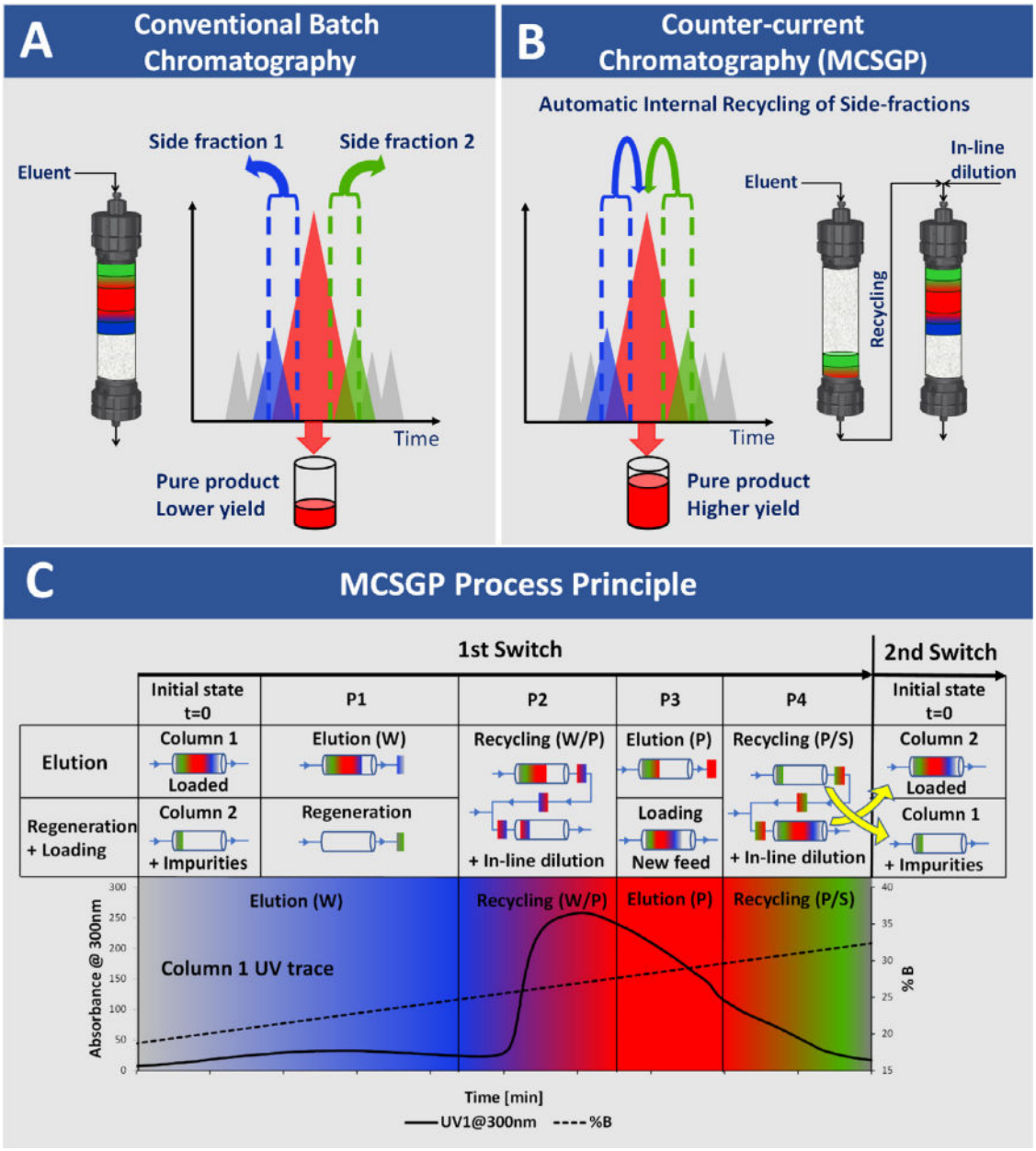

Fig. 1. Introduction to MCSGP. (W) = weakly adsorbing impurities (blue), (P) = product (red), (S) = strongly adsorbing impurities (green), (W/P) = mixture of product and weakly adsorbing impurities, (P/S) = mixture of product and strongly adsorbing impurities. A – Conventional center-cut batch purification produces side fractions of insufficient product purity for pooling leading to product losses; B – By contrast, MCSGP has automatic recycling of side fractions to greatly reduce product losses; C – MCSGP Process Principle showing 1 MCSGP "switch". P1 = phase 1, P2 = phase 2, P3 = phase 3, P4 = phase 4. Columns are operated in parallel during P1 and P3; Columns are interconnected for product recycling in P2 and P4. The position of the columns alternates from switch to switch allowing for continuous operation (yellow arrows indicate column interchange between switches).

55 °C to give the crude, unconjugated oligonucleotide. Removal of excess ammonia, concentration and exchange of the counterion was achieved by ultrafiltration followed by diafiltration against aqueous sodium chloride and water using a Sartoflow ultrafiltration system with 2 kDa Hydrosart membrane (Sartorius). The intermediate was isolated by precipitation with ethanol. For the conjugation reaction, GalNAc acid (1.4 equiv.) was dissolved in *N,N*-dimethylformamide and activated by addition of 2-(5-Norbornene-2,3-dicarboximido)−1,1,3,3-tetramethyluronium tetrafluoroborate (TNTU) (1.4 equiv.). This mixture was added to a solution of the precipitate at 20 wt% in 0.1 M aqueous sodium bicarbonate (pH 8.3). The resulting mixture was stirred for 2 h at ambient temperature after which the title compound was obtained with 77.5 area% purity by RP-HPLC. The crude solution was directly used for purification.

2.2. Batch and MCSGP chromatography

A preparative batch method was carried out as a performance benchmark for comparison to MCSGP as well as to serve as the design template for MCSGP. Batch and continuous chromatography were carried out using the Contichrom CUBE 30, a twin column system for continuous chromatography (ChromaCon AG, A YMC Company). An external column thermostat (Knauer), fitted with two heat exchange cartridges was set to 45 °C and used for all experiments. UV absorbance at 300 nm was recorded by the inter-

Table 1
Materials – Batch vs. MCSGP.

Process	Material	Description
Batch	Column	YMC Triart Prep C8-S (150 × 10 mm ID, S-10 µm, 12 nm)
	Mobile phase A	5 vol% acetonitrile (Merck, 1.00030) / 95 vol% 0.2 M sodium acetate (Sigma-Aldrich, 1.06267) in water
	Mobile phase B	30 vol% acetonitrile / 70 vol% 0.2 M sodium acetate
	Regeneration buffer	78 vol% acetonitrile / 22 vol% 0.2 M sodium acetate
	Feed composition	The synthesis crude was diluted with mobile phase A to a final concentration of 4.5 g/L
	Feed purity	77.5%
MCSGP	Column	2x YMC Triart Prep C8-S (150 × 0.46 mm ID, S-10 µm, 12 nm)
	Mobile phase A	5 vol% acetonitrile / 95 vol% 0.2 M sodium acetate in water
	Mobile phase B	15 vol% acetonitrile / 85 vol% 0.2 M sodium acetate
	In-line dilution buffer	0.2 M sodium acetate in water
	Feed composition	The synthesis crude was diluted in mobile phase A to a final concentration of 2.19 g/L
	Feed purity	77.5%

Table 2
Process parameters overview - Batch vs. MCSGP.

Parameters	Units	Batch process	MCSGP
Run/Cycle time	[min]	47.3	58.9
Column Temperature	[°C]	45	45
Equilibration (P1)	[% ACN]	7.5	5.0
Equilibration (P1)	[CV]	1.84	2.00
Equilibration (P1)	[cm/h]	238	150
Load (Crude) (P3)	[g/L resin]	2.52	1.82
Loading (Crude) (P3)	[CV]	0.56	1.67
Loading (Crude) (P3)	[cm/h]	238	254
Wash after load	[% ACN]	5.0	–
Wash after load	[CV]	0.79	–
Wash after load	[cm/h]	238	–
Gradient Start (P1)	[% ACN]	7.5	7.7
Gradient End (P4)	[% ACN]	15	12.9
Gradient duration (P1 – P4)	[CV]	3.47	3.82
Gradient flow rate (P1 – P4)	[cm/h]	142	142
In-line dilution flow rate (P2)	[cm/h]	–	198
In-line dilution flow rate (P4)	[cm/h]	–	240
Regeneration	[% ACN]	78	–
Regeneration	[CV]	1	–
Regeneration	[cm/h]	238	–
Re-equilibration	[CV]	1.84	–
Re-equilibration	[cm/h]	238	–

nal CUBE 30 UV detectors located directly after each column outlet (UV1@300 nm and UV2@300 nm respectively). Table 1 details the column characteristics, buffer composition and feed composition used for batch and MCSGP runs. Table 2 gives an overview of the method parameters used for batch vs. MCSGP runs. Supplemental Table 1 & 2 detail the specific method parameters used to run batch and MCSGP with the Contichrom CUBE system.

Upon first use, the columns were conditioned for 10 h in Regeneration buffer (6 CV at 9 cm/h). Before starting batch and MCSGP runs, columns were further equilibrated with 6 CV mobile phase A at 45 °C (238 cm/h).

Two notable modifications to the batch method were made for MCSGP:

- The “wash after loading” step was excluded (The strong recycling step effectively substitutes the wash after load step).
- The Regeneration step was applied only at the end of the MCSGP run and not after every elution like in batch (no negative impact on MCSGP process performance was evident).

2.3. Analytics

Analytical HPLC chromatography was carried out using Agilent 1290 Series at 80 °C. An Acquity UPLC Oligonucleotide BEH C18, 50 × 2.1 mm; (1.7 µm) column (Waters) was used with a flow rate of 0.4 mL/min. Detection was carried out at 260 nm. Mobile phase A was composed of 200 mM 1,1,1,3,3,3-Hexafluoro-2-propanol (Sigma-Aldrich, 105,228)/5 mM hexylamine (Sigma-Aldrich, 219,703)/4 mM triethylamine (Sigma-Aldrich, 65,897)/0.004 vol% phosphoric Acid (Sigma-Aldrich, 79,606). Mobile phase B was composed of 10 vol% acetonitrile/90 vol% MeOH (Merck 1.06007). Supplemental Table 3 details gradient parameters used in the method. Samples from the batch & MCSGP fractions were prepared for HPLC analysis by diluting 3-fold with 90 vol% water/10 vol% mobile phase B.

All oligonucleotide concentration measurements were done using a Nanodrop Lite (Thermo Scientific).

3. Results and discussion

A prerequisite for the development of the MCSGP process was the generation of a batch purification process which serves as the MCSGP design starting point as well as a benchmark for process performance comparisons. Since many performance parameters of the batch process are carried over into MCSGP, the batch process should minimally reach the desired target purity threshold, achieve good productivity, and have acceptable buffer consumption. Due to the internal recycling in MCSGP, optimization with respect to yield can be achieved just by adapting a batch process to MCSGP. A batch process with a yield between 20% and 80% is typically a good starting point for MCSGP development.

Fig. 2A shows the UV trace (UV1@300 nm) and % mobile phase B vs. time [min] for the benchmark batch purification run (run parameters are outlined in Tables 1 and 2). A load of 2.52 g/L resin was used (higher loads lead to insufficient product purity, data not shown). Fractions from the product elution were analyzed by HPLC to determine%area product purity (Fig. 2B), and using a Nanodrop to measure total oligonucleotide concentration (g/L). Product and impurity concentrations were calculated as % area abundance in the sample multiplied by the total oligonucleotide concentration (Fig. 2C). Benchmark process performance was then calculated for the pool of fractions with an aggregate purity of 94.22 area% (Fig. 2B, horizontal dotted line = 94% purity threshold), giving a yield of 52.7%, a productivity of 1.3 g/L/h and buffer consumption of 9.59 L/g (Table 3).

The adaptation of the benchmark batch process to MCSGP was facilitated with design software (“MCSGP wizard”). The wizard determines the operating parameters for MCSGP (i.e., gradient concentration and flow rates). The UV trace for the batch run (Fig. 2A), along with data for fraction purity (Fig. 2B) and product concentration (Fig. 2C) were imported into the MCSGP wizard and overlayed. Phase boundaries (P1 – P4) were set as detailed in the legend of Fig. 2. P3 was positioned to achieve a target purity of >94 area% (calculated by the wizard), P2 & P4 were positioned to fully recycle

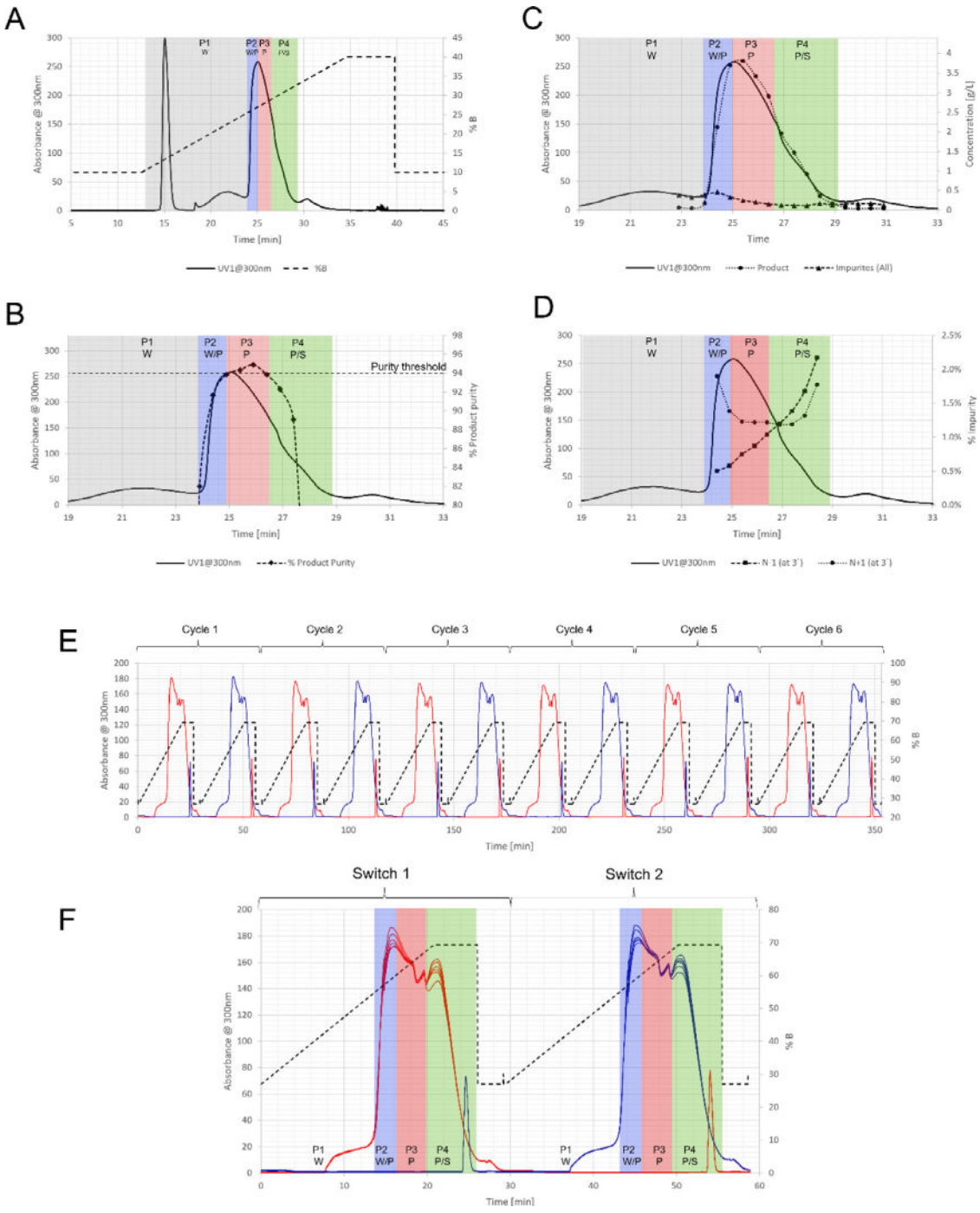

Fig.. 2. A to D - Batch run chromatographic profiles with fraction analysis. Product purity measured by HPLC analysis (area%) and concentration by Nanodrop (g/L). Each dot represents an analyzed fraction. A – Plot showing absorbance UV1@300 nm &%B modifier concentration; B – Plot showing absorbance UV1@300 nm &%area product purity; C – Plot showing absorbance UV1@300 nm & product and impurity concentrations (g/L); D – Plot showing absorbance UV1@300 nm &%area impurities N-1 & $N + 1$. The gradient is split into 4 phases; P1 (W) is the region (gray highlight) containing weakly adsorbing impurities (directed to waste in MCSGP process); P2 (W/P) is the region (blue highlight) with a mixture of product and weakly adsorbing impurities (internal recycling in MCSGP process); P3 (P) is the region (red highlight) with product that achieves purity specification (product elution in MCSGP process); P4 – (P/S) is the region (green highlight) with a mixture of product and strongly adsorbing impurities (internal recycling in MCSGP process). Section boundaries P1 to P4 were used to guide MCSGP design. E & F – MCSGP chromatographic profiles showing absorbance UV1@300 nm plotted vs. time [min], red lines indicate UV signals from column 1 and blue lines indicate elution from column 2. Dotted black line indicates%B modifier concentration. E – Plot showing 6 consecutive MCSGP cycles with 12 product elutions; F – Plot showing an overlay of 6 consecutive MCSGP cycles. Colors indicate phases as described in Fig. 2.

side fractions of lower product purity. When setting the product collection window, it was also important to check the abundance of two critical synthesis impurities (N-1 and N+1). Due to their structural similarity to the target oligonucleotide, these impurities are challenging to remove so it was important to optimally position the product collection window to minimize them (Fig. 2D).

Next, the MCSGP wizard calculated the in-line dilution flow rates necessary to dilute the%B modifier concentration of the eluent back to initial loading conditions (Table 2). This is essential so that as product is recycled from column 1 to column 2, the product can be fully re-adsorbed. Lastly, the loading parameters needed to establish a cyclic steady state were also calculated by the MC-

Table 3
Process performance metrics – Batch vs. MCSGP.

Parameters	Units	Feed	Batch process	MCSGP
Bed height	[cm]		15	2 × 15
Loading flow rate	[cm/h]		238	254
Elution flow rate	[cm/h]		142	142
Washing, cleaning flow rate	[cm/h]		238	150
Product purity	[%]	77.48	94.22	94.16
Impurity N-1	[%]	3.36	0.93	0.87
Impurity $N + 1$	[%]	4.23	1.22	1.41
Yield	[%]		52.70	91.58
Product concentration	[g/L]		3.25	2.71
Productivity	[g/L/h]		1.30	1.33
Load/cycle (Crude)	[g/L]		2.52	1.83
Startup load (Crude)	[g/L]		N/A	2.44
Buffer cons.	[L/g]		9.59	9.22

SGP wizard. Once the design procedure was complete, the MCSGP wizard created 3 methods to be run as a procedure:

- Method 1 was the "Startup" method where the upstream column is pre-loaded with feed like the batch process.
- Method 2 was the main method consisting of 6 cycles of MCSGP.
- Method 3 was a "Shutdown" method consisting of 1 switch with final product elution, but without feeding.

In comparison to the cyclic steady state of MCSGP, the combined performance impact of the "Startup" and "Shutdown" steps have a negative impact on overall MCSGP process performance. This is because the load applied during "Startup" contains more product than is recovered in the final "Shutdown". The final "Shutdown" does not automatically recycle the side fractions, so it is analogous to a batch run in terms of product loss. To maximize the advantage of MCSGP it is therefore ideal to run >20 cycles which will minimize the relative performance impact of the "Startup" & "Shutdown". However, because of the smaller number of cycles run in the study (6 cycles), we restricted the scope of the performance comparison in this paper to the "steady state" i.e., process performance if the number of MCSGP cycles was infinite. While this is the best-case scenario, in practice the process performance of an MCSGP run with more than 20 cycles would be very similar.

Fig. 2E shows the chromatogram of a 6 cycle MCSGP run. Each cycle consists of 2 "switches" with the first switch eluting product from column 1 (UV1@300 nm – red line) and recycling to column 2 and the 2nd switch eluting from column 2 (UV2@300 nm – blue line) and recycling to column 1. Fig. 2F is an overlay of the 6 cycles showing the evolution of the elution profile as the cycles progress. Importantly, the height of the UV profile in the P3 product collection window remains consistent from Cycle 1 to 6 which suggests the material entering the system is in equilibrium with that leaving the system. Changes in the chromatographic profile during early cycles of MCSGP are seen in the P2 & P4 recycling windows as impurities accumulate, taking multiple cycles to reach a steady state. Impurities inside the recycling windows will accumulate until their broadening peaks reach the phase boundary and eventually leave the system via the waste or the product pool. If impurities accumulate in the direction of the P3 product pool, then product purity will decrease cycle to cycle, and product will potentially fall below the desired purity specification. However, by appropriately placing the section borders during the MCSGP design process, impurity accumulation can be avoided or controlled. To determine if product purity was decreasing cycle to cycle, samples were analyzed from all 6 MCSGP cycles by HPLC for purity and by Nanodrop for concentration. Fig. 3A shows that cycle to cycle concentration remains around 2.7 g/L and that total purity is consistently above 94 area%. Fig. 3B confirms that neither N-1 nor $N + 1$ impurities show accumulation during the run. From this, we can conclude that, with respect to product quality, the MCSGP process parameters are well designed. Fig. 3C & D show analytical chromatograms of the final MCSGP product pool (red) for all 6 cycles relative to the input feed material (black) achieving an enrichment of product from 77.5 area% to 94.2 area% purity. Table 3 provides a detailed overview of MCSGP vs. batch process performance for *a* >94 area% purity specification.

Fig. 3E shows a yield vs. purity curve for the batch run compared to the MCSGP operating point. The curve was constructed starting with a data point for the purest fraction with lowest yield. Each additional data point was generated by adding neighboring fractions and recalculating yield and purity until all fractions were included. Fig. 3E demonstrates how the yield / purity tradeoff of the batch process has been overcome in MCSGP, with an operating point in the upper right corner. For equivalent purity, MCSGP outperformed the batch process with respect to yield, 92% vs. 53%, achieving almost complete recovery of the input material (excluding performance impact of "Startup" and "Shutdown" methods). Productivity and buffer consumption of the MCSGP process (1.3 g/L resin/h and 9.2 L/g respectively) were both comparable to the batch process (1.3 g/L resin/h and 9.6 L/g respectively). The load of MCSGP was not optimized and lower than the load value of batch chromatography (1.8 L/g vs. 2.5 L/g). Although load optimization in general was not a part of this study, it was qualitatively determined in batch experiments that small increases in load lead to a further loss in yield, indicating that the binding capacity of the resin was utilized to a great extent. Product concentration was 17% lower in MCSGP so there remains scope for improvement in the method to reduce eluate volumes.

Table 4 shows example scale-up scenarios for the batch and MCSGP processes. Equipment and resin requirements were determined for the reference batch process with a feed input of 3.2 kg/day resulting in product output of 1.3 kg/day, using a single 60 cm inner diameter (ID) column. Two MCSGP setups were compared, the first is designed to process the same input of 3.2 kg/day of feed and the second is designed to produce the same output of 1.3 kg/day of product.

The first MCSGP setup can be exploited to produce 73.6% more product (2270 g/day in MCSGP vs. 1307 g/day in batch) from same quantity of feed material (3200 g/day) or alternatively one can produce the same amount of product, but 42.5% faster with MCSGP. In this scenario, MCSGP would use two columns of 60 cm (ID). Obviously, using the same total resin volume, a batch process could deliver 2614 g/day of product, but with the requirement of 2 × 3200 g/day = 6400 g/day feed.

In the second MCSGP setup, 1.3 kg/day of product can be produced from 42.5% less feed allowing the synthesis to be downscaled by 42.5%. In this comparison, the Batch and MCSGP processes use the same total amount of resin. In this scenario, MCSGP would use two columns of 45 cm ID. The smaller column inner diameter allows use of smaller pump sizes on the skid to obtain the desired linear flow rate of the eluents. Given that MCSGP uses similar equipment with a comparable footprint as a batch chromatography, it is feasible to equip existing facilities.

In addition to improving yields, there are other advantages of MCSGP compared to batch chromatography.

- Firstly, re-chromatography of side fractions is no longer required in MCSGP due to internal recycling. While re-chromatography improves yields in batch chromatography, it greatly decreases overall productivity, increases buffer consumption, and requires extra manual handling and space for storing the pools.
- Secondly, MCSGP provides a potential reduction in the analytical burden of a batch process. In batch chromatography, if the chromatography is very stable, a minimum of three pools are

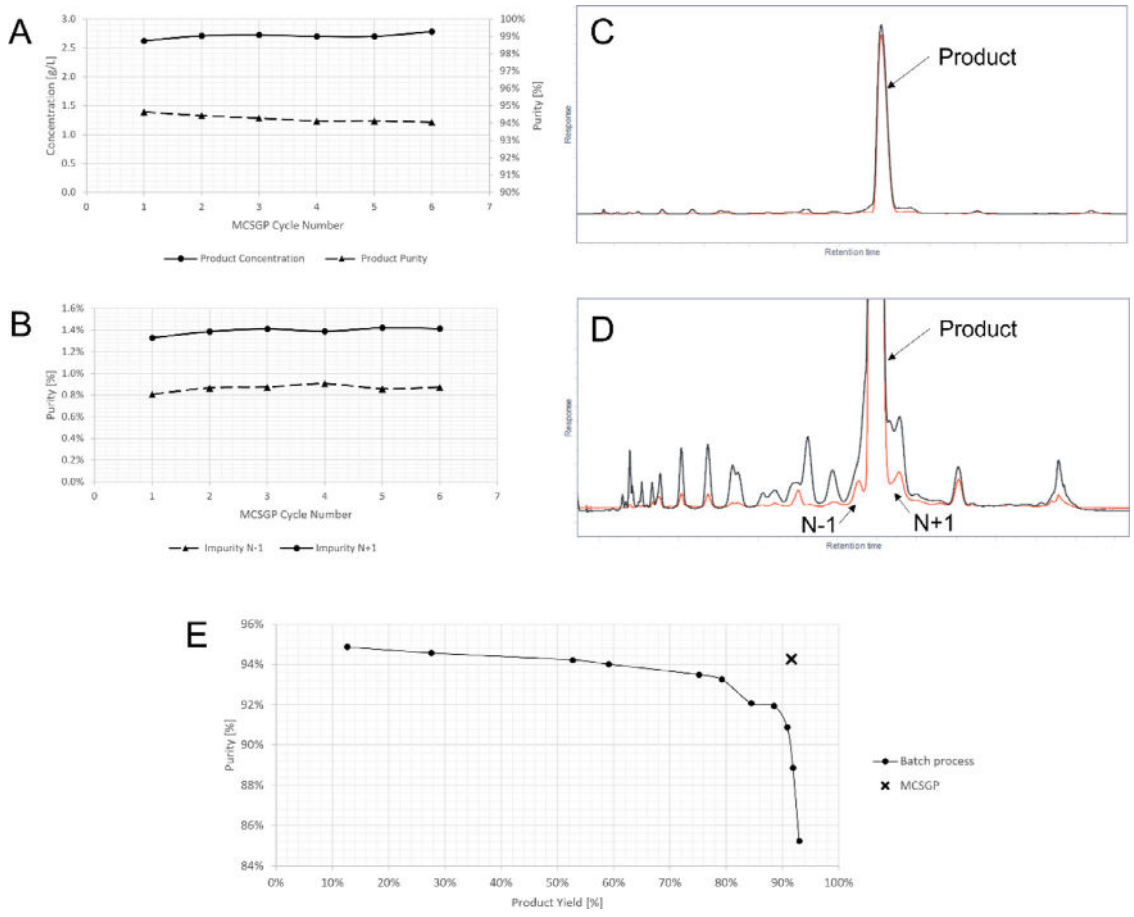

Fig.. 3. MCSGP product quality evaluation. Product purity measured by HPLC analysis (area%) and concentration by Nanodrop (g/L). A – Plot showing cycle to cycle product purity and concentration; B – Plot showing cycle to cycle impurity content (N-1 vs. $N + 1$ impurities); C – Analytical HPLC chromatogram (UV Abs at 260 nm) showing crude (Black line) vs. 6 cycle MCSGP product pool (Red line); D - Zoom of (C). Feed: 77.5%area purity. MCSGP: 94.22%area purity; E - Curve showing the yield / purity tradeoff of the batch chromatographic run vs. the MCSGP run.

Table 4
Scale-up scenario – Batch vs. MCSGP.

Parameter	Batch process	MCSGP with same feed input	MCSGP with same product output
Production amount	1307 g/day	2270 g/day	1307 g/day
Feed input (purity 77.5%)	3200 g/day	3200 g/day	1841 g/day
Column ID	60 cm	2 × 60 cm	2 × 45 cm
Required resin vol	42 L	2 × 35 L	2 × 21 L
Pump size on skid	15 L/min	16 L/min	8 L/min
Daily buffer demand	12,534 L (9.59 L/g)	20,929 L (9.22 L/g)	12,050 L (9.22 L/g)

collected and analyzed (corresponding to W/P; P; P/S pools). However, when product collection is less robust, additional fractions need to be analyzed for every batch run, as well as after re-chromatography. By contrast, typical MCSGP runs generate a single product pool per cycle for analysis, and it may be sufficient to pool and analyze every “n” cycles.

- The third major advantage of MCSGP is a reduced need for human intervention during the production process and therefore reduced personnel requirements.

4. Conclusion

The increasing demand for oligonucleotide-based therapeutics requires new approaches in the chromatographic purification step to enable faster processing, reduction of cost and environmental impact. MCSGP is a potentially superior alternative to batch chromatography for oligonucleotide production. MCSGP delivers higher yields and is also geared towards automation which can result in improved manufacturing speed, elimination of re-chromatography steps, and potentially reduce the burden on quality control. With the potential to downscale the oligonucleotide synthesis step compared to a batch process, MCSGP can help reduce overall material requirements, produces less waste, and save costs. The tradeoff is elevated equipment and validation complexity as an MCSGP setup uses two columns and approximately doubles the amount of hardware components.

In the current study, MCSGP was demonstrated for the reversed phase purification of a GalNAc-cluster-conjugated oligonucleotide, breaking the yield/purity tradeoff of the batch process, and resulting in 73.6% relative yield improvement at 94.2 area% purity. A simplified scale-up estimation showed that performance would facilitate a 42.5% downscaling of the preceding steps, including oligonucleotide synthesis, or alternatively, allow production of the same amount of product 42.5% faster.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Richard Weldon: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Writing – original draft, Project administration. **Jörg Lill:** Conceptualization, Methodology, Writing – review & editing. **Martin Olbrich:** Conceptualization, Methodology, Writing – review & editing. **Pascal Schmidt:** Conceptualization, Methodology, Writing – review & editing. **Thomas Müller-Späth:** Formal analysis, Methodology, Writing – review & editing, Project administration.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2021.462734.

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