Optimization and scale-up of siRNA synthesis

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Introduction

The latest "trend" in oligonucleotide therapeutics is the development of therapeutic siRNA molecules. As its name suggests, RNA interference (RNAi) is a cellular mechanism regulating the expression of genes and also the replication of viruses. The mechanism is mediated by double-stranded small interfering RNA molecules (siRNA). Discovered in 1998, siRNA is the latest biotechnology breakthrough that raises hopes for discovery of the "magic bullet" that would allow the specific targeting of any disease-relevant gene. In theory, siRNA therapeutics can be 100% specific for a particular gene, because the sequences can be selected to interact with one particular mRNA only. Only 6 years after discovery of the therapeutic opportunities in siRNA molecules, two companies, Sirna and Acuity Pharmaceuticals, have already entered clinical trials with siRNA compounds targeted against vascular endothelial growth factor (VEGF) for treatment of age-related macular degeneration (AMD).

siRNA oligonucleotides are short pieces of double-stranded RNA that can associate with cellular proteins to form a protein-based complex called RISC, for **R**NA-**I**nduced **S**ilencing **C**omplex. Within the RISC complex, the two strands of the siRNA become separated, so that they can target complementary sequences in mRNAs involved in a disease. After pairing with an siRNA strand, the targeted mRNA is cleaved and undergoes degradation, thereby interrupting the synthesis of the disease-causing protein. The RISC complex is naturally stable within the cell, enabling siRNAs to cut multiple mRNA molecules consecutively and, therefore, suppressing protein synthesis in a potent and targeted way.

In order for siRNA to become a viable therapeutic technology, highly efficient and reproducible methods must be developed for manufacture that can be scaled up to levels required to meet future commercial demands. This report describes optimization of synthesis, cleavage, and deprotection of a 21-mer siRNA molecule. Optimization was performed at a scale of 95 µmol using a 6.3 ml synthesis column in an ÄKTA™oligopilot 100 system. The optimized process was scaled up 16-fold to 1.52 mmol using a FineLINE™ 70 column in an OligoPilot™ 400 system.

Fig 1. ÄKTA oligopilot 100 was used for the initial optimization and initial scale-up to 380 µmol.

siRNA Sequence

The sequence below was chosen as a model for the optimization and scale-up work. The molecule was chosen because it had been difficult to obtain crude material with acceptable purity after synthesis and deprotection.

5'-GGC CUU CUU UGA GUU CGG UGT-3'

This sequence is both G- and U-rich. Sometimes G-rich sequences can be problematic due to incomplete coupling, and U-rich sequences are susceptible to modifications during deprotection, caused by the reaction of acrylonitrile with the N_3 position of U residues.

Experimental

Synthesis

An ÄKTA oligopilot 100 system was used for optimization at a scale of 95 µmol using a 6.3 ml column. For a four-fold scale-up to 380 µmol, a FineLINE 35 column was used. For the final scale-up to 1.52 mmol, an OligoPilot 400 was used equipped with a FineLINE 70 column.

Reagents used for synthesis, cleavage, and deprotection are listed in Table 1 below.

Cleavage and base deprotection

After synthesis, the solid support was dried for 1 h by application of vacuum and transferred from the column to a Schott bottle. A mixture of methylamine, 33% in ethanol, mixed 1:1 with concentrated ammonium hydroxide (10 ml/g of solid support) was added to the bottle, and the bottle was capped and heated in an oven at 60–65°C for 2 h. The mixture was allowed to cool to room temperature, the cleavage and deprotection mixture was filtered (P3 glass filter), and the solid support was washed with ethanol/water, 1:1 (2×10 ml/g solid support). The combined filtrates were evaporated to dryness using a rotary evaporator.

Desilylation (protocol based on 1 g of solid support)

The crude RNA oligonucleotide, still carrying the 2'-TBDMS groups, was dissolved in DMSO, 1 ml, and transferred to 50 ml Falcon tube. Triethylamine 3HF, 10 ml, was added, the tube was capped, and the mixture was shaken vigorously in order to ensure complete dissolution. The tube was capped and heated in an oven at 60–65°C for 3–3.5 h. The tube was removed from the oven and cooled to room temperature.

Precipitation (protocol based on 1 g of solid support)

The solution containing the completely desilylated oligonucleotide was cooled on dry ice and n-butanol (-20°C), 20 ml, was added carefully in 5 ml portions in order to precipitate the oligonucleotide. The precipitate was filtered and washed with ice-cold n-butanol, 10 ml. The precipitate was finally dissolved in 1 M TEAA (triethylammonium acetate).

Extraction (protocol based on 1 g of solid support)

Alternatively, the sample can be extracted rather than precipitated after desilylation. In this case, after cooling the desilylation solution to room temperature, n-butanol, 20–30 ml, was added and the solution was transferred to a separatory funnel. The bottle used for desilylation was rinsed with n-butanol and 1 M TEAA, and the solutions were transferred to the separatory funnel. TEAA (1 M, 20–30 ml) was added to the separatory funnel. The funnel was shaken vigorously, and the two phases were allowed to separate. The lower water phase containing the RNA was collected, and the pH of the solution was adjusted to 6–7 using ice-cold 0.1 M sodium bicarbonate.

Yield determination

After cleavage and deprotection, the synthesis yields were determined by measuring the absorbance at 260 nm of an aliquot of the crude mixture diluted in water. In order to make the yields comparable for different synthesis scales, they are expressed as A_{260} units/µmol.

Table 1. Reagents used for synthesis, cleavage, and deprotection.

Reagent	Composition/quality	Supplier	Code number
Solid support	Custom Primer Support riboU 80, 81 µmol/g Custom Primer Support T 80s, 76 µmol/g	GE Healthcare GE Healthcare	17-5214-53 17-5253-82
Acetonitrile		Riedel-de Haën or Burdick & Jackson	
Detritylation	3% or 5% DCA in toluene		
Activator	BTT (benzylthiotetrazole) 0.3 M, in ACN	Biosolve or MTC Industries	
Amidite rA^{bz}	TheraPure™ phosphoroamidite, 2'TBDMS	Pierce	27-1903-05
Amidite rC^{ac}	TheraPure phosphoroamidite, 2'TBDMS	Pierce	27-1805-05
Amidite rG^{iso}	TheraPure phosphoroamidite, 2'TBDMS	Pierce	27-1906-05
Amidite rU	TheraPure phosphoroamidite, 2'TBDMS	Pierce	27-1904-05
Capping A	20% NMI in ACN	GE Healthcare	27-9785-03
Capping B	20% Ac_2O, 30% 2,6-lutidine in ACN	GE Healthcare	27-9786-03
Oxidation	50 mM I_2 in pyridine/water 9:1	GE Healthcare	27-9787-03
Deprotection 1	20% Diethylamine in ACN		
Cleavage & deprotection 2	33% Methylamine in ethanol/ mixed 1:1 with concentrated ammonium hydroxide		
Desilylation	Triethylamine 3HF Dimethylsulfoxide		
Precipitation	n-butanol		

HPLC purity analysis

After cleavage and deprotection, the purity of the crude reaction mixtures was analyzed by ion exchange (IEX) HPLC using the conditions shown in Table 2.

Table 2. Conditions used for IEX HPLC analysis.

HPLC system	Agilent 1100
Column	DNA PAC™ PA100
Injection volume	2 µl
Sample conc	20–30 A_{260} units/ml
Buffer A	1 mM Tris, 10 mM $NaClO_4$
Buffer B	1 mM Tris, 300 mM $NaClO_4$
Flow rate	1.0 ml/min
Gradient	1–55% B in 30 min
Column temp	50°C

Optimization

Initially, a number of syntheses were made in a 6.3 ml column in an ÄKTA oligopilot 100 system in order to optimize the synthesis conditions. The primary goal of the initial optimization was to develop a robust, reproducible, and scalable method for work-up after synthesis. The intention was also to try to minimize the amount of N+ impurities eluting just after the full-length product when analyzed by IEX HPLC. These impurities can come from double coupling during the addition of G amidites and/or addition of acrylonitrile to U residues during cleavage and deprotection. The sequence used for these experiments is both G- and U-rich (33% and 43%, respectively), which means that this sequence is especially sensitive to the formation of N+ impurities.

Table 3. Synthesis parameters for initial optimization.

Column volume	6.3 ml
Solid support	Custom Primer Support T 80s, 1.25 g
Support loading	76 µmol/g
Amidite excess	3.5–4 eq
Amidite conc	100 mM
Activator	0.3 M BTT
Amidite/activator ratio	2/3
Oxidation excess	2 eq
Oxidation contact time	1.0 min
Coupling time, G, recycle	12 min
Coupling time, A;C;U; recycle	15 min
DCA conc for detritylation	3%
Diethylamine treatment	Yes

The amount of monomer used in the coupling reaction was not optimized in this investigation. Even though this step is very important from a manufacturing perspective, it was decided to omit it in the current study; it will, however, be addressed in a separate investigation, along with optimization of the nucleoside loading on a Custom Primer Support™. For all work including optimization and scale-up, TheraPure 2´-TBDMS amidites from Pierce were used.

The initial optimization resulted in a synthesis method with the synthesis parameters listed in Table 3 above. It was found that treatment with diethylamine in order to remove the cyanoethyl protecting groups prior to cleavage and base deprotection resulted in a significant reduction of the N+ impurity. Reduction of the coupling time for G residues from 15 min to 12 min also lowered the levels of the N+ impurities caused by double coupling.

Cleavage and base deprotection with a mixture of methylamine, 33% in ethanol, mixed 1:1 with concentrated ammonium hydroxide at 60–65°C for 2 h was found to result in complete cleavage and base deprotection and little or no removal of the 2′-TBDMS groups.

Desilylation was performed with triethylamine 3HF after dissolving the silyl-protected material in a minimal amount of DMSO followed by heating at 60–65°C for 3–3.5 h. Dissolving the oligonucleotide in DMSO prior to treatment with triethylamine 3HF is important in order to obtain a homogeneous reaction mixture during desilylation. If the material is not dissolved in DMSO prior to the treatment with triethylamine 3HF, N+ impurities can be formed as a result of incomplete desilylation.

The final step in the work-up procedure is to remove excess triethylamine 3HF and the reagents used in the desilylation step. Two different methods were developed for this step. The first was to precipitate the RNA from the cooled desilylation mixture with ice-cold n-butanol. After the precipitate was washed with ice-cold n-butanol, it was dissolved in TEAA buffer. The precipitation resulted in a minimal loss of material with a recovery in the 90–95% range. When this method is employed it is critical to use a minimal amount of DMSO in the desilylation reaction. This is to ensure that precipitation will be complete. The second method developed for this step is based on extraction. After the desilylation mixture was cooled, n-butanol was added followed by extraction of the RNA oligonucleotide with 1M TEAA. After the extraction, the pH of the water phase containing the RNA was adjusted to 6–7 using 0.1M ice-cold sodium bicarbonate. Extraction also resulted in a minimal loss of material, and the recovery in this step was in the 95–100% range.

After work-up, the yield of the crude product was determined. Based on the precipitation work-up, the yields obtained were in the range of 120–130 A_{260}/µmol. Analysis of the crude reaction mixture by HPLC after precipitation gave a profile with 72–76% purity. A typical chromatogram based on the optimized, 95 µmol method, is shown in Figure 3.

Scale-up to 380 µmol

Once the synthesis method had been optimized using the 6.3 ml column, a linear scale-up was performed using a FineLINE 35 Oligo column with a bed height of 2.6 cm. A few parameters were slightly changed. The amidite excess was reduced from four to three equivalents. This was based on experience from DNA synthesis. Scaling up normally leads to lower requirements for excess monomer. This is mainly due to less dilution of the coupling reagents when the volume of the coupling reagents is significantly larger than the hold-up volume in the system. Another parameter that was changed compared with the optimization was the amidite concentration, which was increased from 100 mM to 150 mM. This change is also based on experience from DNA synthesis. When working at a lower scale it is essential that the volume of the coupling reagents is significantly larger than the hold-up volume in the system, but as a result of scale-up, as in this case a four-fold scale-up using the same system, the coupling volumes automatically get significantly larger compared with the hold-up volume. Increasing the concentration of the amidite solution when scaling up will lead to an increase in the reaction rate, and this in turn will lead to the possibility of working with a lower monomer excess. Also, the coupling time was reduced compared with the initial optimization runs. For G couplings, potentially sensitive to double coupling due to detritylation, the recycle time was reduced from 12 min to 10 min. For A, C, and U, the recycle time was reduced from 15 min to 13 min. The last parameter that was changed was the DCA concentration in the detritylation solution. It was increased from 3% to 5%. RNA is not sensitive to acid in the same way that DNA can be as a result of acid-catalyzed depurination. Increasing the acid concentration to 5% resulted in a shorter detritylation, and therefore a more concentrated removal of the DMT group.

After work-up, the yield of the crude product was determined. Based on the extraction work-up, the yield was 125 A_{260}/µmol. Analysis of the crude reaction mixture by HPLC after extraction gave a profile with 76% purity. A chromatogram from the 380 µmol synthesis is shown in Figure 4.

Fig 2. OligoPilot 400 was used for the final scale-up to 1.52 mmol.

16-fold scale-up to 1.52 mmol

The synthesis process was then scaled up another four times to 1.52 mmol using a FineLINE 70 column with the same bed height as in the previous run, i.e., 2.6 cm. Based on linear flow rates, the synthesis process was identical to that used in the FineLINE 35 column, The only change was that coupling times were further reduced to 12 min for A, C, and U and 10 min for G.

After work-up, the yield of the crude product was determined. Based on the extraction work-up, the yield was 131 A_{260}/µmol. Analysis of the crude reaction mixture by HPLC after extraction gave a profile with 76% purity. A chromatogram from the 1.52 mmol synthesis is shown in Figure 5.

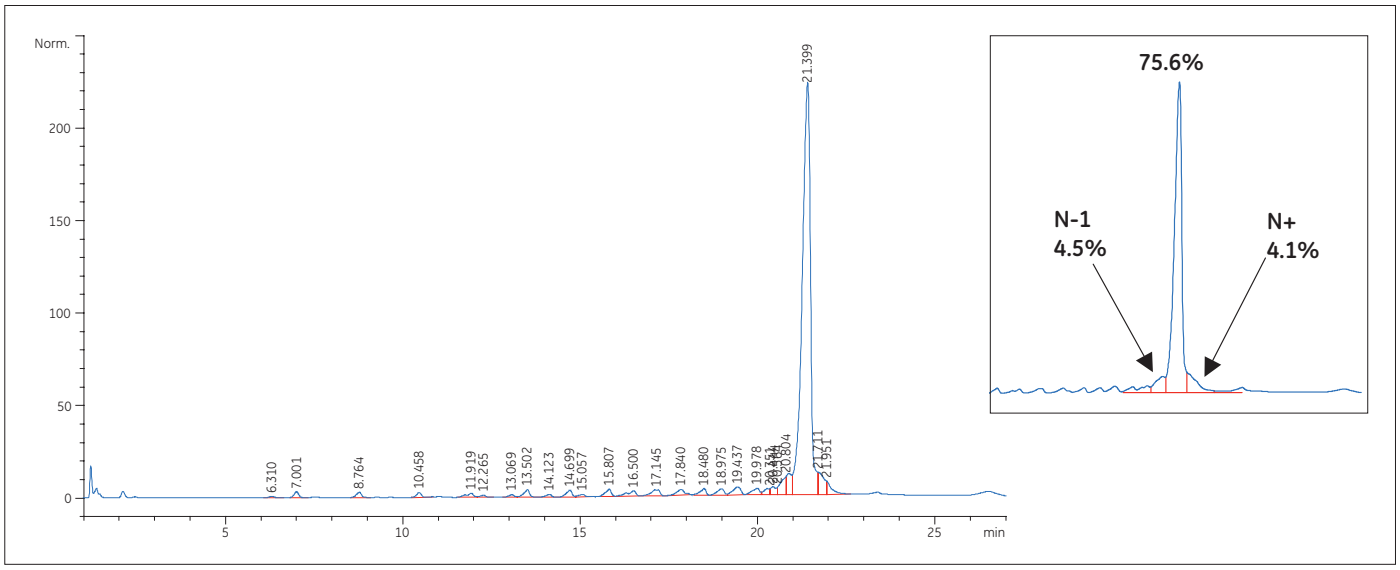

Fig 3. HPLC analysis of crude mixture after 95 µmol synthesis and work-up using precipitation. Integration of the N-1 and N+ impurities was performed as indicated in the figure.

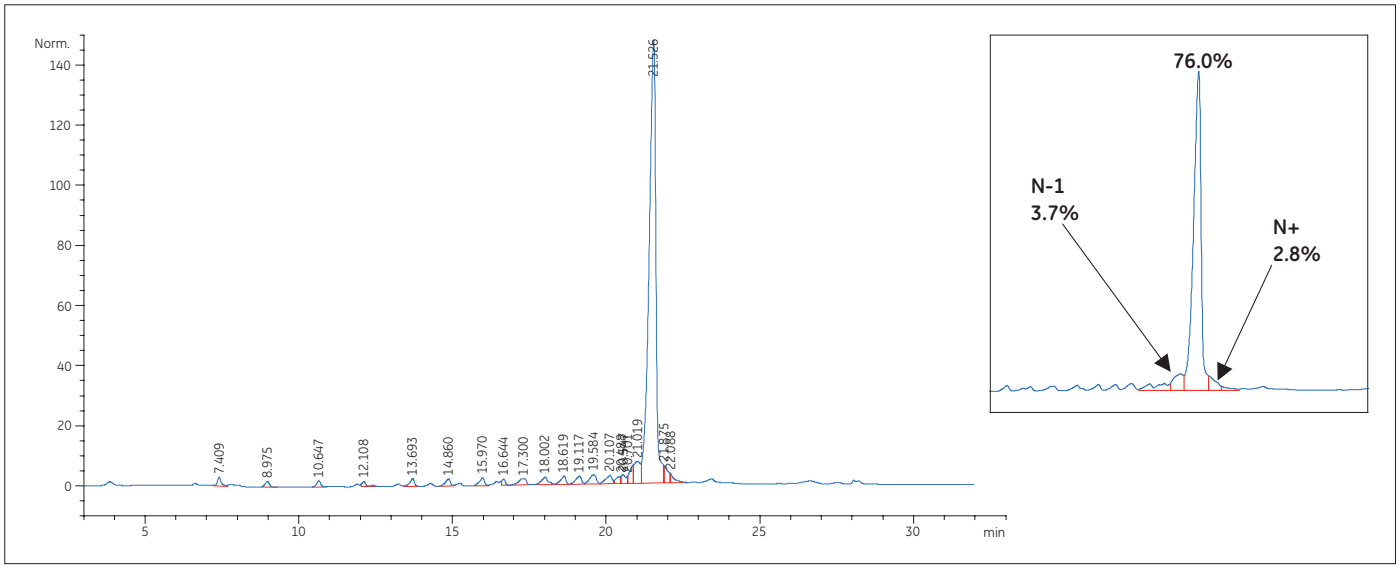

Fig 4. HPLC analysis of crude mixture after 380 µmol synthesis and work-up using extraction. Integration of the N-1 and N+ impurities was performed as indicated in the figure.

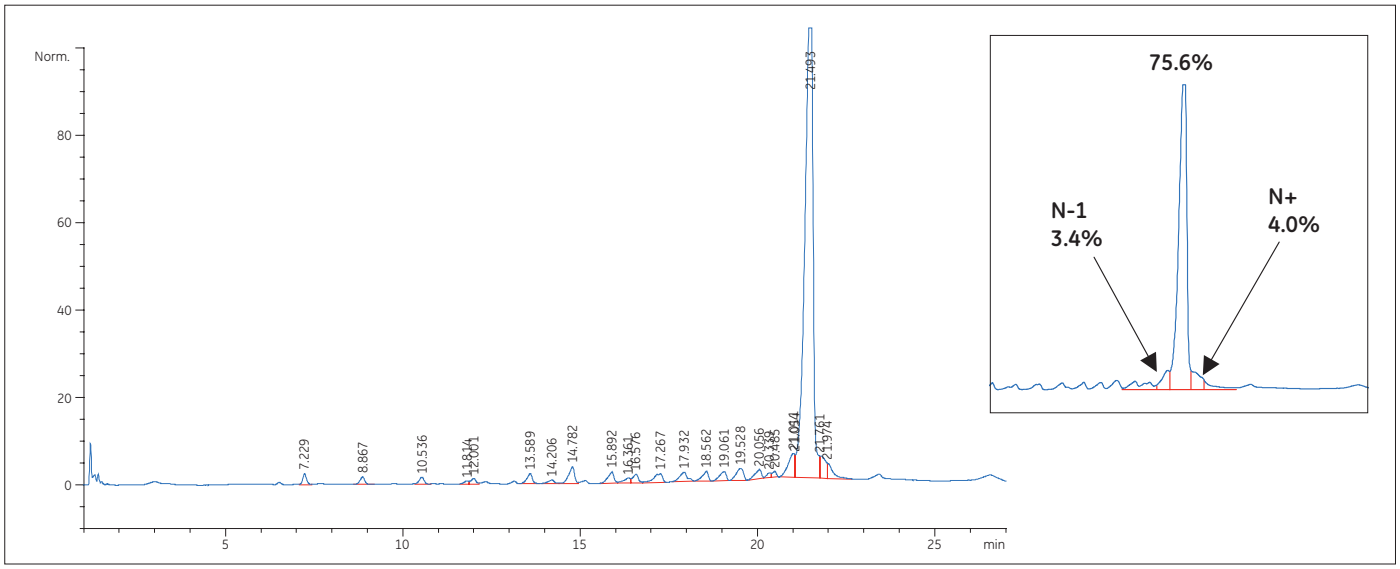

Fig 5. HPLC analysis of crude mixture after 1.52 mmol synthesis and work-up using extraction. Integration of the N-1 and N+ impurities was performed as indicated in the figure.

Conclusions

The synthesis process for making an unmodified siRNA molecule has been scaled up successfully from 95 µmol to 1.52 mmol. A highly efficient procedure has also been developed involving either precipitation or extraction as part of the work-up after desilylation. The purity and yields were consistently high and found to remain so regardless of the synthesis scale. For the particular siRNA sequence chosen for this work, the yields were in the 125–135 A_{260}/µmol range and the purity was approximately 76% based on IEX HPLC.

Based on this work, it can be concluded that scale-up of unmodified RNA can be accomplished very efficiently. Successful scale-up of DNA synthesis has been demonstrated up to 750 mmol. Based on this knowledge, together with the work presented here, it can be concluded that further scale-up of RNA synthesis to meet the demand based on the development of siRNA-based therapeutics will be possible without having to compromise either yield or purity.

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