Versatile synthesis of oligodeoxyribonucleotide–oligospermine conjugates

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A protocol for the rapid, automated synthesis of oligospermine–oligonucleotide sequences is described. To this end, a protected spermine phosphoramidite derivative was synthesized in six steps from spermine and used as the fifth synthon in an oligonucleotide synthesizer. Parameters were optimized to reach greater than 95% coupling yields. Cationic oligonucleotides show enhanced hybridization and strand invasion properties, and hence are an alternative to conventional oligonucleotides for molecular biology, diagnostic and potential therapeutic applications. A multi-gram-scale synthesis of the spermine phosphoramidite allowing several hundred coupling steps takes 2–3 weeks. Oligonucleotide synthesis and purification takes approximately 3 d.

INTRODUCTION

Oligonucleotides are at the heart of some of the most powerful molecular biology techniques, such as PCR, DNA chips and *in situ* hybridization. Moreover, oligonucleotides would represent a generic, yet very selective, class of drugs if they were able to cross cell membranes[1–3]. Many chemical modifications have been developed, including thiophosphate, peptide nucleic acid, locked nucleic acid and morpholino oligonucleotides, to improve their properties. Further modifications are necessary, in particular for therapeutic uses. Owing to the polyanionic nature of oligonucleotides, conjugation to a polycationic moiety is the rational approach to decrease charge repulsion and thus improve hybridization properties. Moreover, polycations are known to carry oligonucleotides into cells, hence their conjugates are expected to behave similarly. Various synthetic approaches for introducing cations into oligonucleotides have been described, including phosphate backbone replacement, nucleotide modifications or end-conjugation, for which enhanced hybridization, strand invasion and eventually cell penetration were observed.

Among cation-modified oligonucleotides, those leaving the oligonucleotide moiety intact retain mismatch discrimination and remain substrates of nucleic acid–processing enzymes[4–13]. Moreover, molecular biology applications require rapid on-demand synthesis of oligonucleotide sequences, and this requirement was not fulfilled by previous synthetic approaches.

We therefore developed a versatile synthesis of oligospermine–oligonucleotides on the basis of machine-compatible phosphoramidite chemistry[14]. Spermine units were chemically introduced during oligonucleotide synthesis using an appropriately protected spermine phosphoramidite (**1** in **Fig. 1**). This robust chemistry allowed us to achieve rapid and high-yield coupling of the spermine phosphoramidite. However, conventional deprotection in hot ammonia led to some oligospermine hydrolysis. Decomposition was avoided by using commercially available UltraMILD phosphoramidite monomers, for which the deprotection step can be carried out at room temperature (25 °C). The resulting deprotected oligonucleotides were purified by HPLC and characterized by mass spectrometry. A series of 10–20-mer oligonucleotides with up to 20 spermine units appended was prepared, including several fluorescein-labeled oligonucleotides. Although we describe only oligonucleotides with a 5′ block of spermines, spermine(s) can be introduced at any position in the sequence. In our design, each spermine unit contains four secondary amino groups and a phosphate residue; that is, it adds up to three net cationic charges. As anticipated, oligospermine conjugation had a strong positive effect on duplex stability ($\Delta T_m = 2.2^\circ$ per spermine unit; see ref. 14).

MATERIALS

REAGENTS

- Spermine (Fluka, cat. no. 85590)
- 2-Mesitylenesulfonyl chloride, 99% (Aldrich, cat. no. M7707)
- Sodium hydride, dispersion, approximately 60% wt/wt in mineral oil (Fluka, cat. no. 62863) ! **CAUTION** Contact with water liberates extremely flammable gases.
- *tert*-butyl(4-iodobutoxy)dimethylsilane, 95% (Aldrich, cat. no. 51202-8)
- Hydrogen bromide, minimum 33% in glacial acetic acid (Riedel de Haën, cat. no. 02206) ! **CAUTION** Causes severe burns.
- Phenol, 99% (Aldrich, cat. no.18545-0) ! **CAUTION** Toxic, corrosive.
- Triethylamine, 99.5% (Aldrich, cat. no. 47128-3)
- 2-Cyanoethyl-diisopropylchloro phosphoramidite (Aldrich, cat. no. 30230-9)
- Trifluoroacetic anhydride, 99% (Aldrich, cat. no. 10623-2) ! **CAUTION** Reacts violently with water; harmful if inhaled.
- 4,4′-Dimethoxytritylchloride (Aldrich, cat. no. 10001-3)
- Pyridine (Fluka, cat. no. 82704) ! **CAUTION** Highly flammable.
- Dichloromethane stabilized with amylene (Carlo Erba)
- Acetone
- Dimethylformamide (DMF) pure for synthesis (SDS, cat. no. 0340216)
- Ethyl acetate pure for synthesis (Carlo Erba, cat. no. 0500229)
- Toluene
- Fontainebleau sand (VWR)
- Silica gel, 40–63 μm (Merck Geduran, Kieselgel Si 60)
- Argon
- Ammonium hydroxide
- NaOH, analytical (VWR-Prolabo, Normapur, cat. no. 28244.295)
- $NaHCO_3$, analytical (VWR-Prolabo, Normapur, cat. no. 27778.293)
- Magnesium sulfate, anhydrous (Alfa Aesar, cat. no. L13739)
- Anhydrous acetonitrile (ACN; 2.5 l, SDS, cat. no. 63521; 100 ml, Glen Research, cat. no. 40-4050)
- Nucleoside-functionalized controlled pore glass supports: Pac-dA-lcaa-CPG (Glen Research, cat. no. 20-2601); Ac-dC-lcaa-CPG (Glen Research,

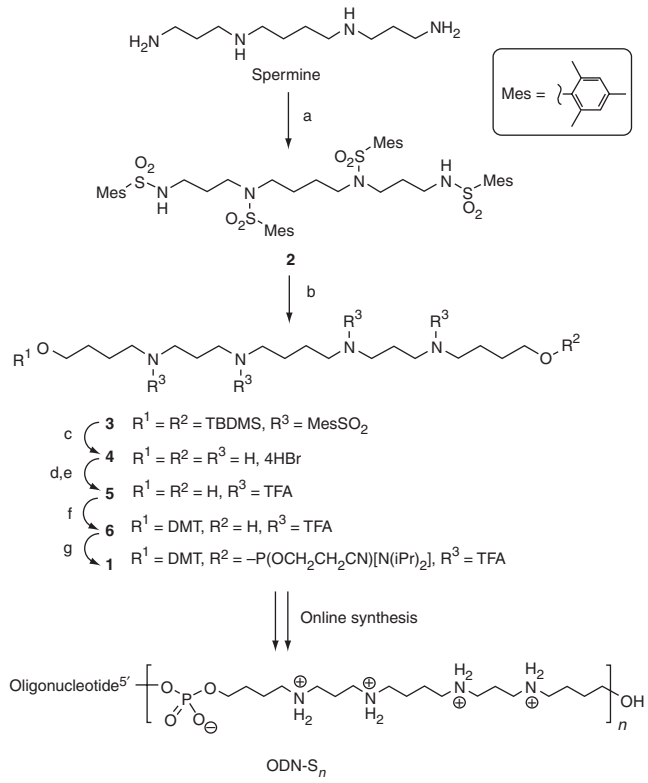

Figure 1 | Synthesis of spermine synthon **1** and of oligonucleotide–spermine conjugates. Reagents/yield: (a) $MesSO_2Cl$/NaOH/80% (ref. 15) (b) NaH/TBDMSO$(CH_2)_4$I/76%; (c) HBr/phenol/quant.; (d) TFA_2O/pyridine; (e) $NaHCO_3$/MeOH/61% for two steps; (f) DMTCl/pyridine/43%; (g) $ClP(OCH_2CH_2CN)[N(iPr)_2]/NEt_3$/73%.

cat. no. 20-2015); iPr-Pac-dG-lcaa-CPG (Glen Research, cat. no. 20-2621); T-lcaa-CPG (Glen Research, cat. no. 20-2231)
- Deoxyribonucleoside phosphoramidites: Pac-dA-CE phosphoramidite (Glen Research, cat. no. 10-1601-05E); Ac-dC-CE phosphoramidite (Glen Research, cat. no. 10-1015-C5); iPr-Pac-dG-CE phosphoramidite (Glen Research, cat. no. 10-1621-05E); 5′-DMT-T-CE phosphoramidite (Glen Research, cat. no. 10-1030)
- 5′-Fluorescein phosphoramidite (Glen Research, cat. no. 10-5901)
- Oxidizing solution: 0.02 M iodine in tetrahydrofuran (THF), water and pyridine (Glen Research, cat. no. 40-4132)
- Anhydrous wash: anhydrous ACN (Glen Research, cat. no. 40-4050)
- Activator: sublimed ^1H tetrazole in anhydrous ACN (Glen Research, cat. no. 30-3100)
- Cap mixA: 5% (vol/vol) phenoxyacetic anhydride in THF (Glen Research, cat. no. 40-4212-52)
- Cap mixB: 10% (vol/vol) 1-methylimidazole in THF/pyridine (Glen Research, cat. no. 40-4122)
- Deblocking mix: 3% trichloroacetic acid in dichloromethane (Glen Research, cat. no. 40-4140)
- Triethylamine acetate, 2.0 M HPLC grade (Glen Research, cat. no. 60-4110-52)
- Nitrogen
- Aqueous ammonia, 28% (Aldrich)
- Cyclohexane
- Methanol
- Diethyl ether
- Trifluoroacetic acid, 99% (Alfa Aesar, cat. no. L06374) ! **CAUTION** Harmful if inhaled; causes severe burns.

EQUIPMENT
- Septum
- Separatory funnel
- Fluted filter paper (Whatman)
- Sintered glass funnel with standard ground glass joints and porosity 2
- TLC aluminum sheets, silica gel 60 F-254 (Merck, cat. no. 1.05554.0001)
- Rotary evaporator (Buchi)
- Glass chromatographic columns, 5 cm i.d. × 63.5 cm long and 2 cm i.d. × 63.5 cm long
- Addition funnel
- Pipetman P1000 and P200 (Gilson)
- DNA synthesizer (Expedite 8900)
- Fraction collector Model 2110 (Bio rad)
- Hemolysis tubes, 7 ml
- Volumetric flasks, 5 ml
- Cary 100Bio UV-Vis spectrophotometer (see EQUIPMENT SETUP)
- Disposable plastic syringe, 1 ml, and needle, 0.8 × 40 mm^2 (Terumo)
- Membrane filters, 0.45 µm (Millipore, cat. no. HAWP04700)
- Reversed-phase cartridges for DNA isolation (PolyPak, DMT-ON)
- Sephadex NAP-10 G25, DNA grade (see EQUIPMENT SETUP)
- PolyPak II barrel (Glen research, cat. no. 60-4100-30) (see EQUIPMENT SETUP)
- Disposable syringe filter, ∅ 15 mm; pore ∅ 45 µm (Macherey-Nagel, cat. no. 729023)
- High pressure/performance liquid chromatograph with a UV and γ-detector connected in series (see EQUIPMENT SETUP)
- HPLC micro-syringe: Hamilton Microliter 700 Series syringe, Model 705 and 750, point style 3
- Anion-exchange HPLC column

EQUIPMENT SETUP

HPLC setup Column: VA 50/7.7 Nucleogel SAX 1000-8.
Mobile phase: a: NH_3 0.1 M; b: NH_3 0.1 M, NaCl 1 M.
Flow rate: 4 ml min^{-1}.
Detector: 260, 280 and 494 nm.
Turn the HPLC UV detector on and equilibrate the column by allowing at least 30 ml solvent (30% b/70% a) to flow through the column.

NAP-25 Sephadex preparation Remove the cap and pour off the excess liquid. Cut the end of the column tip. Equilibrate the gel with 25 ml of deionized water and allow the water to completely enter the gel bed.

UV setup Turn the UV instrument on and wait for equilibration. Measure the background signal of the reference solvent.

PolyPak II reverse-phase cartridge preparation DMT-ON oligonucleotides were purified on a PolyPak II reverse-phase cartridge. Flush the cartridge with 4 ml ACN and then with 4 ml of 2 M triethylammonium acetate.

PROCEDURE

Synthesis of N^1,N^4,N^9,N^{12}-tetrakis(mesitylenesulfonyl)spermine (2)

1| In a 500-ml round-bottomed flask containing a Teflon-coated magnetic stir bar, weigh out 8.0 g (39 mmol) of spermine and dissolve it in 100 ml of dichloromethane. Turn the magnetic stirrer on[15].

2| Dispense 100 ml of 2 N NaOH (7.8 g of NaOH per 100 ml of water) into the reaction flask. Stir and cool the mixture at 0 °C using a water/ice bath.

3| Fit the flask with a rubber septum and insert an argon inlet and a vent consisting of disposable syringe needle. Weigh out 34.5 g (160 mmol) of mesitylenesulfonyl chloride and add it by small portions into the flask over 10 min using a spatula. Recap with the septum and turn on the argon tank so that the reaction mixture stays under argon atmosphere.

■ **PAUSE POINT** Stir overnight at room temperature under argon.

4| Transfer the mixture to a separatory funnel and isolate the aqueous phase. Extract it with three 50-ml portions of dichloromethane. Wash the pooled dichloromethane solution, extracting it with 50 ml of aqueous $NaHCO_3$ (1 M). Dry the organic layer with the addition of 25 g of anhydrous magnesium sulfate. Stir gently for 15 min.

5| Filter the mixture under gravity through a fluted filter paper on a glass funnel to remove magnesium sulfate and collect the filtrate into the flask. Evaporate the dichloromethane using a rotary evaporator at room temperature under low vacuum.

6| Recrystallize the white-foamed residue in 150 ml of cold acetone. Separate the crystals on a sintered glass filter to get 24.2 g of the desired white solid with 67% yield. Dry it using a vacuum pump.

Synthesis of N^1,N^{12}-bis[4-(*tert*-butyldimethylsilyloxy)butyl]-N^1,N^4,N^9,N^{12}-tetrakis(mesitylenesulfonyl)spermine (3)

7| Fit a 250-ml round-bottomed flask with a Teflon-coated magnetic stir bar containing 24 g (26 mmol) of (**2**) dissolved in 50 ml of DMF with a rubber septum. Then insert an argon inlet and a vent consisting of disposable syringe needle. Turn on the argon flow.

8| Turn the magnetic stirrer on and cool the reaction flask to 0 °C with a water/ice bath.

9| Weigh out 2.6 g (64 mmol) of NaH (60% dispersion in mineral oil) in a beaker. Pour 5 ml of pentane into the beaker. Sonicate the mixture and remove the supernatant. Repeat twice and dry the solid for several minutes under low vacuum (in a desiccator). Add it gradually by small portions to the reaction mixture over 10 min using a spatula.
! CAUTION Remove the septum during the addition as H_2 gas develops upon NaH addition. Note that NaH becomes highly reactive against moisture after oil removal by pentane washing. Note also that H_2 gas is highly flammable (even explosive in conjunction with atmospheric O_2); make sure to run this reaction in a fume hood and that there are no open flames in the vicinity.

10| Stir at room temperature for 30 min under argon. Add 16.7 ml of *tert*-butyl(4-iodobutoxy)dimethylsilane to the reaction mixture using a Pasteur pipette.
■ **PAUSE POINT** Stir overnight at room temperature under argon.

11| Dilute the reaction mixture with water/dichloromethane (250 ml/250 ml). Transfer the mixture in a separatory funnel and separate the organic phase. Wash the aqueous phase with two 125-ml portions of dichloromethane. Wash the pooled dichloromethane solutions by extraction with 100 ml of $NaHCO_3$ (1 M) and dry with the addition of 20 g of anhydrous magnesium sulfate. Stir gently for 15 min at room temperature.

12| Filter the mixture under gravity through a fluted filter paper on a glass funnel to remove the magnesium sulfate. Evaporate the solvent using a rotary evaporator under high vacuum at 25 °C to remove residual DMF.

13| Pack a chromatography column (5 × 17 cm^2 length) with silica gel using a 4:1 mixture of cyclohexane and ethyl acetate. Cover the top of the column further with a layer of sand (1 cm thick). Load the crude mixture on top of the silica bed using a Pasteur pipette. Elute the column under light pressure with 4:1 cyclohexane and ethyl acetate.

14| Collect 50-ml fractions. Identify fractions containing the desired product (expected to be fractions 7–20) by TLC by eluting thin-layer gel plates on aluminum backing with 4:1 cyclohexane and ethyl acetate ($R_f = 0.3$).

15| Evaporate the pooled fractions containing the product under low vacuum at 25 °C using a rotary evaporator and dry the clear oil using a high-vacuum pump. Recover 33 g with 97% yield.

Synthesis of N^1,N^{12}-bis(4-hydroxybutyl)spermine tetrahydrobromide (4)

16| In a 1-l round-bottomed flask containing a Teflon-coated magnetic stir bar, dissolve 28 g (22 mmol) of (**3**) in 220 ml of dichloromethane. Turn the magnetic stirrer on.

17| As phenol is easier to manipulate in liquid form, heat the container using a water bath at 45 °C. Measure 75 ml (860 mmol) phenol and add it to the reaction mixture. Cool the mixture to 0 °C with an ice bath. Dispense dropwise 217 ml (1.26 mol) of HBr (33% in glacial acetic acid) over 1 h using an addition funnel.
■ **PAUSE POINT** Stir overnight at room temperature.

18| In the morning, add 150 ml of cold water (10 °C) to the reaction mixture. Transfer the mixture to a separatory funnel. Isolate the organic phase and extract it with two 50-ml portions of water. Wash the pooled aqueous solution by extraction with two 100-ml portions of dichloromethane.

19| Evaporate the aqueous phase using a rotary evaporator under high vacuum at 30 °C.
Note: Install an acid trap made of solid NaOH to avoid acid damage to the pump.

20| Wash the orange residue from Step 19 with 1.5 l of diethyl ether until the wash-through loses its orange color and the residue becomes solid. Evaporate the residual ether to get 12.5 g of an orange solid in 87% yield.
■ **PAUSE POINT** Dry the solid further under high vacuum overnight.

Synthesis of N^1,N^{12}-bis(4-hydroxybutyl)-N^1,N^4,N^9,N^{12}-tetrakis(trifluoroacetyl)spermine (5)
21| In a 500-ml round-bottomed flask containing a Teflon-coated magnetic stir bar, make a suspension of 12.4 g (18 mmol) of (**4**) in a mixture of dichloromethane/pyridine (80 ml/35 ml). Turn the magnetic stirrer on.

22| Cool the reaction mixture to 0 °C using an ice bath. Dispense dropwise 38 ml (270 mmol) of trifluoroacetic anhydride over 30 min using an addition funnel. Stir for 3 h at room temperature.
Note: Add trifluoroacetic anhydride very slowly as harmful white trifluoroacetic acid fumes develop upon addition.

23| Cool the reaction mixture to 0 °C. Add 80 ml of cold water and 80 ml of dichloromethane. Separate the organic phase and wash the aqueous phase with two 50-ml portions of dichloromethane. Wash the pooled dichloromethane solution by extraction with three 50-ml portions of water. Dry the organic layer over 20 g of anhydrous magnesium sulfate. Allow the mixture to stir for 15 min.

24| Filter the mixture under gravity through a fluted filter paper on a glass funnel to remove the magnesium sulfate. Evaporate the solvent using a rotary evaporator under low vacuum at 25 °C to get a clear oil.

25| Dissolve the oil from Step 24 in 100 ml of methanol. Weigh out 80 mg of $NaHCO_3$ and add it to the reaction mixture.
■ **PAUSE POINT** Stir overnight at room temperature.

26| Evaporate the solvent using a rotary evaporator under low vacuum at 25 °C.

27| Pack a chromatography column (5 × 17 cm^2 length) with silica gel using a 95:5 mixture of dichloromethane and methanol. Cover the top of the column further with a layer of sand (1 cm thick). Load the crude mixture on top of the silica bed using a Pasteur pipette. Elute the column under light pressure with 95:5 dichloromethane and methanol (1 l), then 93:7 dichloromethane and methanol (300 ml).

28| Collect 50-ml fractions. Identify fractions containing the desired product (expected to be fractions 14–24) by TLC by eluting thin-layer gel plates on aluminum backing with 4:1 ethyl acetate and cyclohexane ($R_f = 0.25$).

29| Evaporate the pooled fractions containing the product using a rotary evaporator under low vacuum at 25 °C and dry the clear oil with a high-vacuum pump. Recover 8.56 g of a yellowish oil (80% yield).

Synthesis of N^1-[4-(dimethoxytrityloxy)butyl]-N^{12}-(4-hydroxybutyl)-N^1,N^4,N^9,N^{12}-tetrakis(trifluoroacetyl)spermine (6)
30| In a 100-ml round-bottomed flask containing a Teflon-coated magnetic stir bar, dissolve 8.5 g (12 mmol) of (**5**) in a mixture of dichloromethane and pyridine (20:23 ml). Turn the magnetic stirrer on. Fit the flask with a rubber septum and then insert an argon inlet and a vent consisting of a disposable needle. Turn on the argon flow.

31| Cool the reaction mixture to 0 °C using an ice bath. Weigh out 4.33 g (13 mmol) of 4,4′-dimethoxytrityl chloride and add it gradually in small portions to the reaction mixture using a spatula over a 10-min period. The solution turns orange. Stir under argon at room temperature for 3 h.

32| Remove the solvent using a rotary evaporator under high vacuum at 25 °C. Then perform a co-evaporation by dissolving the residue in 20 ml toluene to remove the remaining pyridine. Evaporate the solvent using a rotary evaporator under high vacuum at 25 °C. Repeat co-evaporation step twice.

33| Pack a chromatography column (5 × 17 cm^2 length) with silica gel using dichloromethane. Cover the top of the column further with a layer of sand (1 cm thick). Load the crude mixture on top of the silica bed using a Pasteur pipette. Elute the column under light pressure successively with (i) dichloromethane (250 ml; 50 ml fractions 0–5), (ii) dichloromethane 90%/acetone 10% (600 ml; 6–20), (iii) dichloromethane 80%/acetone 20% (600 ml; 21–35), (iv) dichloromethane 90%/methanol 10% (300 ml; 36–46) and (v) dichloromethane 80%/methanol 20% (250 ml; 46–55).

34| Collect 50-ml fractions. Identify fractions containing the desired product (expected to be fractions 16–30) by TLC by eluting thin-layer gel plates on aluminum backing with dichloromethane 90%/acetone 10% ($R_f = 0.25$).

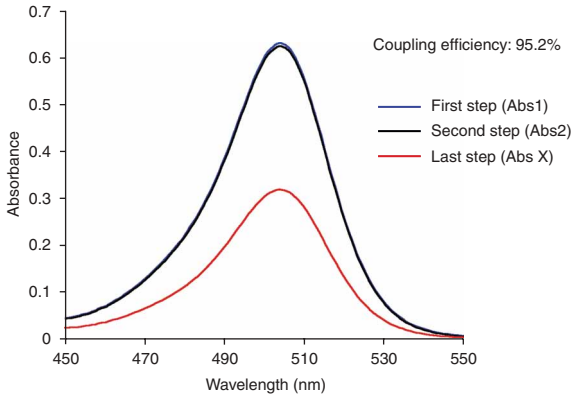

Figure 2 | UV data for determination of coupling efficiency: the amount of DMT group released after each coupling cycle was assessed by measuring the absorbance at 503 nm.

35| Evaporate the pooled fraction containing the desired product using a rotary evaporator under low vacuum at 25 °C and dry the clear oil with a high-vacuum pump. Recover 3.75 g of a yellowish oil (40% yield).

Synthesis of phosphoramidite spermine (7)

36| In a 25-ml round-bottomed flask containing a Teflon-coated magnetic stir bar, dissolve 1.18 g (1.14 mmol) of (**6**) in 5 ml of dichloromethane. Turn the magnetic stirrer on.

37| Dispense first 315 µl of triethylamine and then 280 µl of 2-cyanoethyl-diisopropylchloro phosphoramidite into the reaction flask using a micropipette. Stir at room temperature for 30 min.

38| Pack a chromatography column (2 × 17 cm^2 length) with silica gel using a 60:40 mixture of cyclohexane and ethyl acetate containing 5% vol/vol triethylamine (200 ml).
▲ **CRITICAL STEP** The synthesized phosphoramidite spermine (**7**) is acid-sensitive. Addition of 5% of triethylamine is necessary to deactivate the silica gel and prevent product decomposition.

39| Allow the column to equilibrate with a 60:40 mixture of ethyl acetate and cyclohexane plus 0.1% volume of triethylamine (200 ml). Load the crude mixture on top of the silica bed using a Pasteur pipette. Elute the column under light pressure with 250 ml of a 60:40 mixture of ethyl acetate and cyclohexane containing 0.1% vol/vol triethylamine.

40| Collect 20-ml fractions. Identify fractions containing the desired product (fractions 4–8) by TLC by eluting thin-layer gel plates on aluminum backing with 8:2 dichloromethane and acetone (R_f = 0.5).

41| Evaporate the pooled fractions containing the product using a rotary evaporator under low vacuum at 25 °C and dry the clear oil with a high-vacuum pump. Recover 0.9 g of a transparent oil (65% yield).
■ **PAUSE POINT** Product **7** can be stored in the –20 °C freezer for up to 3 months.

Oligonucleotide synthesis

42| Prepare required volumes (approximately 0.13 ml for coupling) of 58–67 mM phosphoramidite solutions in anhydrous ACN by dissolving the four natural phosphoramidites A, C, G and T (0.5 g in 10 ml), the modified spermine phosphoramidite (0.5 g in 6 ml) and the fluorescein phosphoramidite (100 µmol in 1.3 ml; only for fluorescein-labeled oligonucleotides)[16].

43| Place the reagents in the synthesizer and prime them twice.

44| Start the automated solid-phase synthesis from the appropriate solid support–filled column (1-µmol scale) with DMT-ON mode (i.e., no final deprotection keeping the dimethoxytrityl moiety (–DMT) on the last coupled nucleotide). Elongate the desired oligonucleotide chain using the four natural phosphoramidites A, C, G and T, the modified spermine phosphoramidite and/or the fluorescein phosphoramidite, depending on whether you want a labeled oligonucleotide. Allow 20-min coupling for each spermine and fluorescein phosphoramidite coupling step. For the natural nucleic bases, 7 min is sufficient. Collect DMT fractions resulting from each deprotection step.

45| After completion of the assembly, check the coupling efficiency by measuring the absorbance of the collected DMT fractions at 503 nm. Transfer the first collected fraction into a 5-ml volumetric flask. Complete to 5 ml with dicloromethane using a Pasteur pipette. Pipette 250 µl and transfer it to another 5-ml volumetric flask. Complete to 5 ml with a solution of 3% trichloroacetic acid in dichloromethane (vol/vol) with a Pasteur pipette. Measure the absorbance at 503 nm with dichloromethane as the reference solvent. The absorbance is usually between 0.6 and 0.9 for 1-µmol-scale synthesis. Repeat step with the following collected fractions. The first two and the last fractions are sufficient to estimate the coupling efficiency. Calculate the coupling efficiency using the following formula: coupling efficiency = $(Abs2/Abs1)^{1/step\ number}$.

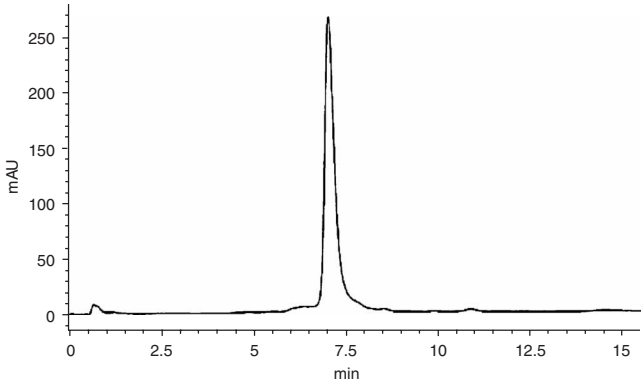

Figure 3 | HPLC absorption trace at 260 nm versus time (min) obtained after purification of 5′-SSSSSS-GAA-GAT-GGT-GCG-3′ using option B.

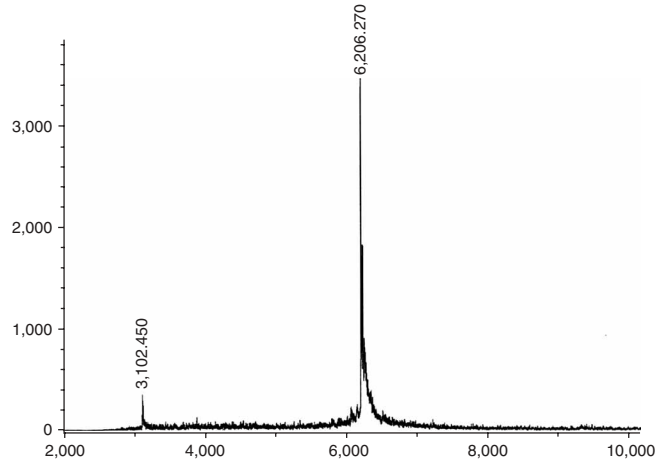

Figure 4 | Matrix-assisted laser desorption/ionization (MALDI) spectrum (matrix: hydroxypicolinic acid). Calculated mass of 5′-SSSSSS-GAA-GAT-GGT-GCG-3′: 6,198, found: 6,206.

46| Remove the column and transfer the solid phase into a screw-capped Eppendorf vial by cutting the end of the column. Push 0.7 ml of 28% ammonia through the emptied column to wash it.

47| Vortex the mixture, close the vial well and incubate overnight at 25 °C.

48| Take the supernatant using a syringe. Fit it with a filter (∅ 15 mm; pore ∅ 45 µm) and transfer the solution into an Eppendorf vial. Add 100 µl of 28% ammonia and vortex to wash the remaining solid support. Repeat washing twice.
▲ **CRITICAL STEP** An excess of solid support in the syringe may block the filter when passing through it: solid support should remain at the bottom.

Oligonucleotide purification

49| Carry out the purification of the oligonucleotide by HPLC, implementing either Option A or Option B, depending on whether the oligonucleotide is labeled with fluorescein. As the fluorescein moiety we used has no DMT group, fluorescent oligonucleotides must be purified using the HPLC technique (A, which is also applicable for non-fluorescent oligonucleotides). Non-fluorescent oligonucleotides are purified using PolyPak II reverse-phase cartridges (B), which is our preferred method as it is less time and cost consuming.

(A) Fluorescein-labeled oligonucleotide

(i) Inject 5 µl of the crude mixture using a 50-µl micro-syringe to determine the retention time of the desired fluorescein-labeled oligonucleotide. It should come up at 494 nm (the expected absorption wavelength for fluorescein).

(ii) Withdraw 300 µl of the mixture in a 500-µl micro-syringe. Inject the mixture onto an anion-exchange HPLC column. Elute, gradually increasing the ratio of b from 30 to 100% over 30 min at a flow rate of 4 ml min^{-1}.

(iii) Collect the fraction corresponding to the fluorescent oligonucleotide at the predetermined retention time and evaporate the solvents under low vacuum at room temperature using a rotary evaporator. Repeat step until there is no mixture left to purify. Note that, owing to the high-salt-containing HPLC eluant, the mixture requires desalting.

(iv) Dissolve the salt-containing fluorescent oligonucleotide in 2 ml of deionized water. Load it on the Sephadex NAP-25 cartridge's gel bed. Allow the sample to enter the gel completely. Add 0.5 ml of deionized water (so that the combined volumes equal 2.5 ml). Allow the water to enter the gel completely.

(v) Elute the purified sample with 3.5 ml of deionized water and collect 0.5-ml fractions.

(vi) Pool the fluorescent fractions and remove the water using a rotary evaporator under low vacuum at room temperature. Dissolve the residue in 500 µl of deionized water. If the product is not completely soluble in pure water, use a 50 mM ammonium hydroxide solution.

(vii) Check the purity by HPLC with a 5-µl injection of the purified oligonucleotide [see Step 49A(ii) for elution conditions]. Dilute the oligonucleotide solution 50 times and measure the concentration by UV absorbance at 260 nm using triethylamine acetate 10 mM as the reference solvent. Calculate the extinction coefficients using the empirical formula: $E_{260} = [(8.8 \times nT) + (7.3 \times nC) + (11.7 \times nG) + (15.4 \times nA)] \times 0.9$ (spermine part does not absorb at 260 nm)[16].

■ **PAUSE POINT** The solution of the purified oligonucleotide can be stored in the −20 °C freezer for up to 3 months.

(B) Non-fluorescein-labeled oligonucleotide

(i) Add three parts of deionized water to one part of the non-labeled oligonucleotide. Load the sample onto the PolyPak II cartridge, collect the eluted fraction and push it again through the cartridge.

(ii) Flush the cartridge successively with (a) 4 ml of ammonium hydroxide (1/20), (b) 4 ml of deionized water, (c) 4 ml of 2% TFA and (d) 4 ml of deionized water. Elute the purified oligonucleotide with 3 ml of 20% ACN

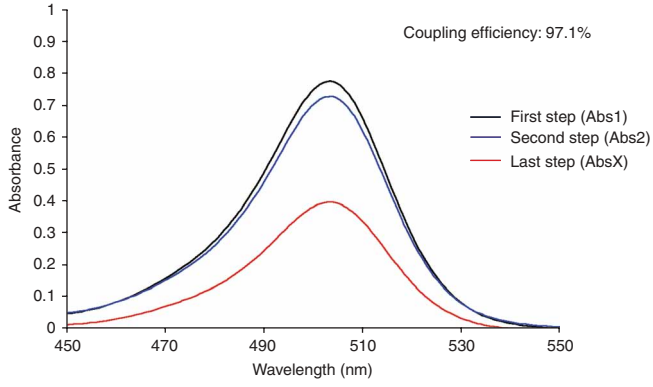

Figure 5 | UV data for determination of coupling efficiency.

in water. Collect 0.5-ml fractions. Check each fraction for fluorescence quenching under UV lamp irradiation by spotting a drop on a F-254 silica TLC plate.

(iii) Pool the fluorescence-quenching fractions and remove the solvent using a rotary evaporator under low vacuum at room temperature. Dissolve each sample into 500 µl of deionized water. If the product is not completely soluble in pure water, use a 50 mM ammonium hydroxide solution instead.

(iv) Check the purity by HPLC with a 5-µl injection of the purified oligonucleotide [see Step 49A(ii) for elution conditions]. Dilute the oligonucleotide solution 50 times and measure the concentration by UV absorbance at 260 nm using triethylamine acetate 10 mM as the reference solvent.

■ **PAUSE POINT** The solution of the purified oligonucleotide can be stored in the −20 °C freezer for up to 3 months.

? TROUBLESHOOTING

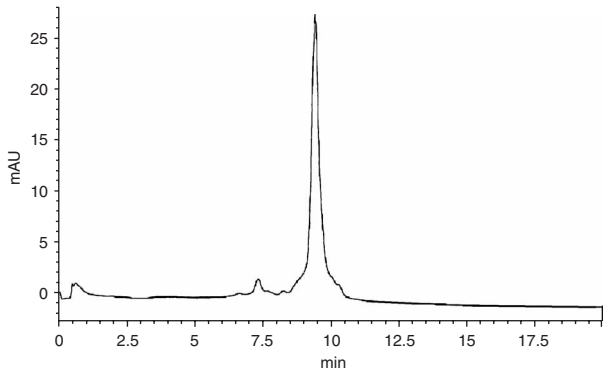

Figure 6 | HPLC absorption trace at 260 nm versus time (min) after purification of 5′-F-SSSSS-TGG-AAG-ATG-GAA-CCG-CTG-GA-3′ following Option A.

● **TIMING**

Steps 1–3, 18 h; Step 4, 45 min; Steps 5 and 6, 1 h; Steps 7–10, 18 h; Steps 11 and 12, 1 h; Steps 13–15, 4 h; Steps 16 and 17, 18 h; Steps 18–20, 4 h; Steps 21 and 22, 3 h 30; Steps 23 and 24, 1h; Step 25, 15 h; Steps 26–29, 4 h; Steps 30 and 31, 3 h 45; Step 32, 30 min; Steps 33–35, 5 h; Steps 36 and 37, 45 min; Steps 38–41, 5 h; Steps 42 and 43, 30 min; Step 44 and 45, depends on the sequence; Step 46–48, 18 h; Step 49A(i), 35 min per injection; Step 49A(ii), 15 min; Steps 49A(iii–v), 30 min; Step 49A(vi), 45 min; Step 49B(i–iii), 30 min; Step 49B(iv), 30 min

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 1**.

TABLE 1 | Troubleshooting table.

Problem	Possible reason	Solution
Low yields for oligonucleotide synthesis	Reagent alteration owing to moisture contamination	Replace reagents with fresh ones
Difficulties dissolving oligonucleotides in deionized water	Balance between positive and negative charges may cause insolubility	Use a 50 mM ammonium hydroxide solution instead
Oligonucleotides stick to the PolyPak II cartridge after the final elution		Use an NH_3 1/20 80%/ acetonitrile 20% solution as the final eluant

ANTICIPATED RESULTS

Synthesis of 5′-SSSSSS-GAA-GAT-GGT-GCG-3′

Purification was carried out via Option B (Step 49) first using a 20% ACN/80% water solution and then using a 20% ACN/80% [5% aqueous NH_3 in water] solution for the final elution, because the oligonucleotide sticks to the Polypak II cartridge. The synthesis, performed using the DMT-ON mode, involved 20 min coupling time for each spermine phosphoramidite and 7 min for each nucleotide phosphoramidite.

In **Figure 2** are reported the absorption spectra of the first two and the last collected fractions in the automated oligonucleotide synthesis. Through these data we were able to estimate a coupling efficiency of 95.2% for the synthesis (see Step 45).

HPLC data reported in **Figure 3** give a sense of the high degree of product purity we obtained after implementation of Step 49B.

Finally, **Figure 4** reports the matrix-assisted laser desorption/ionization (MALDI) spectrum of the oligonucleotide–oligospermidine conjugate.

Synthesis of 5′-F-SSSSS-TGG-AAG-ATG-GAA-CCG-CTG-GA-3′

Purification was carried out via Step 49A (HPLC). The mobile phase was: Buffer a: NH_3 0.1 M; Buffer b: NH_3 0.1 M, NaCl 1 M. Gradient of b was from 30 to 100% over 30 min at a flow rate of 4 ml min^{-1}.

The synthesis, performed using the DMT-ON mode, involved 20 min coupling time for each spermine and fluorescein phosphoramidite and 7 min for each nucleotide phosphoramidite.

In **Figure 5** are reported the absorption spectra of the first two and the last collected fractions in the automated oligonucleotide synthesis. Through these data we were able to estimate a coupling efficiency of 97.1% for the synthesis (see Step 45).

HPLC data reported in **Figure 6** give a sense of the high degree of product purity we obtained after implementation of Step 49B.

Finally, **Figure 7** reports the MALDI spectrum of the fluorescent oligonucleotide–oligospermidine conjugate.

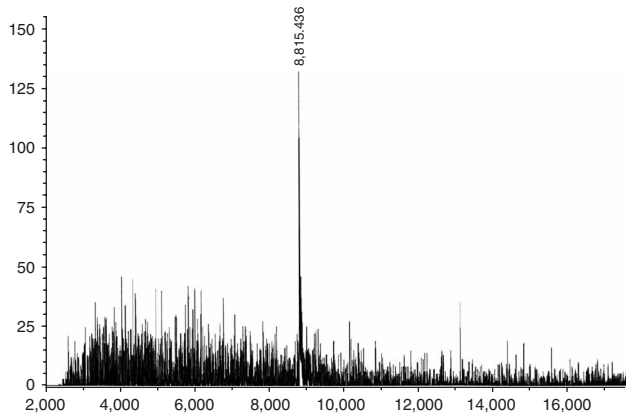

Figure 7 | Matrix-assisted laser desorption/ionization (MALDI) spectrum (matrix: hydroxypicolinic acid). Calculated mass of 5′-F-SSSSS-TGG-AAG-ATG-GAA-CCG-CTG-GA-3′: 8,813, found: 8,815.

ANALYTICAL DATA

(**2**) ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.29 (m, 4H), 1.64 (m, 4H), 2.29 (s, 9H), 2.57 (sd, 18H), 2.77 (m, 4H), 3.04 (m, 4H), 3.18 (m, 4H), 4.86 (t, $J = 7$ Hz, 2H), 6.93 (d, $J = 4$Hz, 8H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 21.1, 23.5, 24.8, 28.0, 39.8, 43.3, 45.5, 76.8, 77.5, 78.1, 132.2, 133.5, 134.2, 139.3, 140.3, 142.5, 143.0, 143.2.

(**3**) ^{1}H NMR (200 MHz, $CDCl_3$): δ 0.01 (s, 12H), 0.89 (s, 18H), 1.35 (m, 12H), 1.45 (m, 4H), 2.32 (s, 12H), 2.57 (s, 24H), 3.05 (m, 16H), 3.46 (m, 4H), 6.96 (m, 8H).

(**4**) ^{1}H NMR (200 MHz, D_2O): δ 1.61–1.73 (m, 12H), 2.05 (m, 4H), 3.07 (m, 16H), 3.57 (t, $J = 6$Hz, 4H).

(**5**) ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.59–1.95 (m, 18H), 3.42 (m, 16H), 3.70 (m, 4H).

(**6**) ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.62–2.05 (m, 17H), 3.1 (m, 2H), 3.4 (m, 16H), 3.7 (m, 2H), 3.79 (s, 6H), 6.84 (m, 4H), 7.20–7.43 (m, 9H).

(**1**) ^{1}H NMR (200 MHz, $CDCl_3$): δ 1.23 (m, 12H), 1.64–2.01 (m, 16H), 2.7 (t, $J = 6$ Hz, 2H), 3.12 (m, 2H), 3.41–3.74 (m, 20H), 3.82 (s, 6H), 6.88 (m, 4H), 7.24–7.47 (m, 9H); ^{31}P NMR (81 MHz, $CDCl_3$): δ 148.01.

COMPETING INTERESTS STATEMENT The authors declare competing financial interests (see the HTML version of this article for details).

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