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Automated parallel synthesis of 5′-triphosphate oligonucleotides and preparation of chemically modified 5′-triphosphate small interfering RNA

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Dedicated to Professor Fritz Eckstein on the occasion of his 80th birthday.

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ABSTRACT

A fully automated chemical method for the parallel and high-throughput solid-phase synthesis of 5′-triphosphate and 5′-diphosphate oligonucleotides is described. The desired full-length oligonucleotides were first constructed using standard automated DNA/RNA solid-phase synthesis procedures. Then, on the same column and instrument, efficient implementation of an uninterrupted sequential cycle afforded the corresponding unmodified or chemically modified 5′-triphosphates and 5′-diphosphates. The method was readily translated into a scalable and high-throughput synthesis protocol compatible with the current DNA/RNA synthesizers yielding a large variety of unique 5′-polyphosphorylated oligonucleotides. Using this approach, we accomplished the synthesis of chemically modified 5′-triphosphate oligonucleotides that were annealed to form small-interfering RNAs (ppp-siRNAs), a potentially interesting class of novel RNAi therapeutic tools. The attachment of the 5′-triphosphate group to the passenger strand of a siRNA construct did not induce a significant improvement in the in vitro RNAi-mediated gene silencing activity nor a strong specific in vitro RIG-I activation. The reported method will enable the screening of many chemically modified ppp-siRNAs, resulting in a novel bi-functional RNAi therapeutic platform.

1. Introduction

Biophosphates[1] are a class of biomolecules in all living organisms and are of interest to researchers in synthetic chemistry and biology, biochemistry, diagnostics and therapeutics. Among synthetic biophosphate analogues, nucleoside and oligonucleotide (ON) 5′-triphosphates are essential biochemical and therapeutic tools. They are antiviral[2] and anticancer[3] inhibitors, are substrates for polymerase chain reactions, nucleic acids ligation reactions,[4–6] are useful for structural and mechanistic studies,[7] result in immune response stimulation,[8] and are intermediates in the enzymatic synthesis of m^{7}G-5′-ppp capped RNAs.[9–12]

RNA interference (RNAi), mediated by small interfering RNA (siRNA),[13] is a powerful gene regulation mechanism that has become an established biotechnological tool and a promising therapeutic platform.[14,15] In addition to the gene-silencing activity of siRNAs, their immunostimulatory properties are an independent functional feature.[16] In fact, double-stranded RNA (dsRNA), and siRNAs in particular, are known to activate the immune system.[17,18] The modulation of their desired and undesired immunostimulatory effects could provide agents that exhibit both potent gene silencing and directed immunostimulation that might be useful in treatment of viral infections or cancers.[19,20] Biochemical[21,22] and structural[23,24] studies have shown that 5′-triphosphate dsRNAs activate the retinoic acid inducible gene I (RIG-I), a receptor that functions in the innate immune response.[25] These substrates resemble siRNAs as they are short (19–25 base pairs) dsRNAs but have a 5′-triphosphate group that is essential for enzymatic recognition.[8] Furthermore, a recent report described the potential therapeutic use of bi-functional in vitro generated 5′-triphosphate siRNA (ppp-siRNA) as a single molecule that resulted in both RIG-I activation and RNAi-mediated gene silencing to inhibit melanoma proliferation in vivo.[20] Synthetic 5′-triphosphate RNAs are therefore proven tools of crucial biological importance that have a number of advantages over the triphosphate RNAs generated by in vitro transcription. These advantages include high purity, reproducible yield (independent of RNA sequence and terminal nucleotide), potential for large-scale production, and ability to incorporate chemical modifications into the RNA.[26]

An efficient method for the chemical synthesis of 5′-triphosphate RNAs should therefore ideally consist of an automated,

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high-throughput parallel solid-phase synthesis that takes advantage of the established and largely available automated procedures for solid-phase ON synthesis[27,28] with introduction of the triphosphate group in a robust, automated and high-yielding fashion. However, the lack of a robust procedure for triphosphate synthesis has been a bottleneck. The 5′-triphosphate ON synthesis is usually associated with low to moderate conversion (10–50%),[30–32] and hence the isolation of the pure full length product (FLP) triphosphate ON can vary in difficulty from a substantial challenge to an impossible task. As a step towards the establishment of an efficient solid-phase chemical synthesis of triphosphate ONs, we recently reported synthesis of 5′-triphosphate ONs performed on solid support using the manual 'syringe' technique.[29]

Encouraged by our earlier efforts, we focused on establishing a fully automated procedure for the synthesis of 5′-triphosphate ONs, experimentally as close as possible to the common procedures used in standard DNA and RNA synthesis.[27] It is indeed of particular interest and elegance to consider a unique automated procedure, in which the ON would be first assembled in standard fashion, followed by the subsequent and uninterrupted attachment of the 5′-triphosphate moiety, using the same instrument. In this paper, we describe a method for the automated parallel solid-phase parallel and high-throughput synthesis of 5′-triphosphate and 5′-diphosphate ONs. The chemically modified single-stranded 5′-triphosphate RNAs synthesized following this approach were used for the preparation of representative triphosphorylated siRNAs (ppp-siRNAs) for which the cell-based biological profiles were investigated.

2. Results

2.1. Automated solid-phase synthesis of 5′-triphosphate oligonucleotides using a DNA/RNA synthesizer

We initiated our study using a standard ABI-394 DNA/RNA synthesizer. As a model, we chose to study the automated solid-phase synthesis of a 10-mer 2′-deoxythymidine oligonucleotide $(dT)_{10}$ with a 5′-triphosphate. Starting from 5′-*O*-dimethoxytrityl (DMTr) protected 2′-deoxythymidine, attached to a controlled pore glass (CPG) support, a standard trityl-off oligonucleotide synthesis cycle, employing 3′-phosphoramidite monomers (**2**), was performed, yielding the solid-supported $(dT)_{10}$ oligonucleotide (**4**, Fig. 1).

The four-step triphosphate synthesis sequence was programmed as an 'end procedure' allowing the uninterrupted introduction of the 5′-triphosphate group (**11a**) after completion of the ON synthesis (Fig. 1). Based on our previously established manual coupling sequence for introducing 5′-triphosphates on ONs,[29] we used four bottle positions to dispense in succession the reagents necessary for the triphosphate synthesis, including (Fig. 1) (1) 5′-phosphitylation of the ON **4**,[33] using diphenyl phosphite (**5**); (2) hydrolysis of the resulting phenyl 5′-*H*-phosphonate diester to the corresponding 5′-*H*-phosphonate monoester **9**, using bicarbonate buffer (**6**); (3) silylation and oxidation of the 5′-*H*-phosphonate **9** onto an activated 5'-phosphorimidazolidate **10**,[34] using the mixture of *N*,*O*-bis(trimethylsilyl)acetamide (BSA), bromotrichloromethane and imidazole (**7**); and (4) nucleophilic substitution of the imidazole from **10** by the pyrophosphate anion, using the suitable tributylammonium salt of pyrophosphate (**8a**, Fig. 1). Two modifications relative to the previously reported procedure were necessary for successful implementation on the oligonucleotide synthesizer: (1) acetonitrile was added as a co-solvent for reagents **6** and **8**; (2) in reagent **7**, the highly toxic and carcinogenic[35] carbon tetrachloride was replaced with the safer bromotrichloromethane.[36] After completion of the automated synthesis, release from the solid support and removal of the ON protective groups was performed using standard conditions (Fig. 2).[27]

The desired 5′-triphosphate (ppp-$(dT)_{10}$, **12**, Table 1) was obtained in quality similar to superior to that obtained with the manual procedure, as demonstrated by the HPLC analysis of the crude products (Fig. 3A and 3B). The near quantitative conversion of $(dT)_{10}$ (Fig. 3C) to the corresponding triphosphate was achieved (Fig. 3B). The repeated additions of fresh reagents (**5**–**8**) to the synthesis column during the automated synthesis significantly reduced the overall triphosphate synthesis time from 24.5 h[29] to 8.8 h (see Supplementary data, Supplementary Table S3). In addition, reagents (**5**–**8**) were stable and efficiently re-used upon storage for at least one week on the synthesizer and several weeks at 4 °C, indicating shelf stability and compatibility of these reagents with automated DNA/RNA synthesis.

Since no automated procedures for the preparation of any class of synthetic triphosphates had been described previously, we investigated whether our synthetic method (Fig. 1) presents any advantages or limitations over the existing chemical methods for solid-phase triphosphate synthesis.[30,32] The state-of-the-art procedure for synthesizing triphosphate ONs on solid support involves the use of 2-chloro-1,3,2-benzodioxaphosphorin-4-one[37] as the 5′-phosphitylating reagent, followed by coupling with pyrophosphate and iodine-promoted oxidation.[30,31,38] We automated this manual procedure[30,38] and compared it with our synthesis method. The use of 2-chloro-1,3,2-benzodioxaphosphorin-4-one on an ABI-394 synthesizer was a challenge due to precipitation of a dark mass shortly after this reagent's solution in pyridine and dioxane was delivered to the synthesis column the first time. This precipitation caused blockage of the delivery lines and the column inlet. Moreover, the highly reactive nature and moisture sensitivity of this chlorophosphite reagent presented difficulties to its use in the automated synthesis. HPLC analysis of the crude products indicated that the automated synthesis employing the 2-chloro-1,3,2-benzodioxaphosphorin-4-one reagent was clearly inferior to our automated procedure (Fig. 3B vs Fig. 3D). Attempts to optimize yield by fine-tuning reagent delivery to the column and waiting times or by changing solvent systems did not improve the overall synthesis efficiency of the 2-chloro-1,3,2-benzodioxaphosphorin-4-one route.

2.2. Parallel high-throughput synthesis of 5′-triphosphate oligonucleotides using a high-throughput DNA/RNA synthesizer

To further assess the utility of our automated synthetic approach for its application in high-throughput parallel solid-phase ON synthesis, we performed a synthesis using a high-throughput MerMade-192 DNA/RNA synthesizer. Employing a 96-well plate format, we synthesized twelve ppp-$(dT)_{10}$ ONs (**12 A1**–**A12**, Table 1) in a parallel fashion on one 12-well row of the 96-well plate on the 1 μmol scale. After releasing the products from the solid support (Fig. 2), the IEX-HPLC profiles of the crude compounds showed efficient conversions of the starting $(dT)_{10}$ ONs to their corresponding twelve triphosphates (**12 A1**–**A12**), with at minimum of 76% purity in the crude mixture (Fig. 4).

2.3. Automated parallel synthesis of 5′-triphosphate RNAs

Using a four-column ABI-394 DNA/RNA synthesizer, we synthesized two different 13-nucleotide (nt) RNA sequences (Table 1) in both 5**'**-non triphosphorylated (**13**, **14**) and 5′-triphosphorylated (**15**, **16**) forms. Synthesis was performed on the four columns using 1 μmol of the commercially available RNA 'ultra mild' CPG solid supports and the corresponding commercially available 2′-O-TBDMS[39] 'ultra mild' phosphoramidites[40] (Fig. 1). The automated triphosphate synthesis was then uninterruptedly executed on the two columns dedicated to the synthesis of **15** and **16**, immediately after completion of the ON synthesis as previously discussed

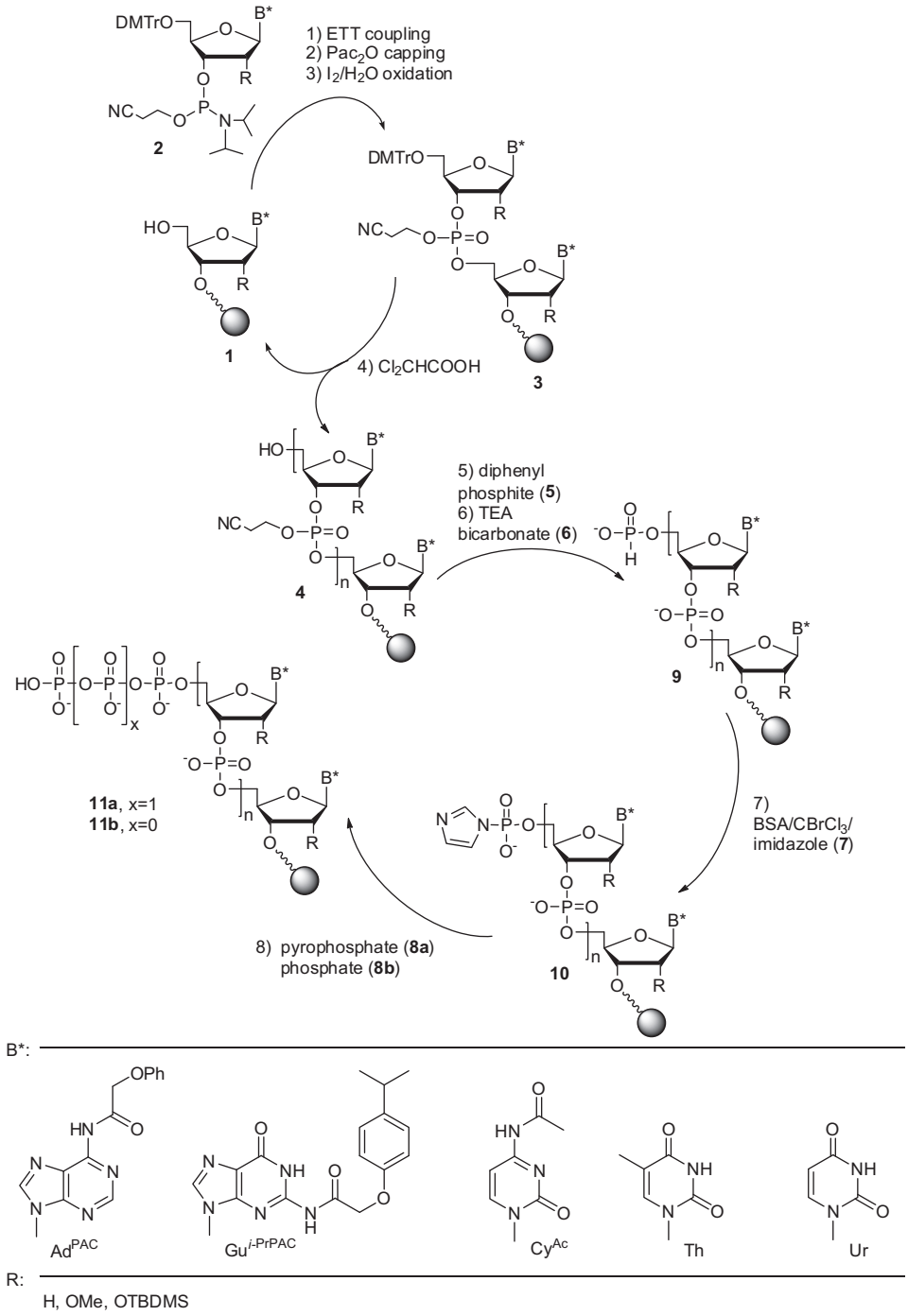

Figure 1. Solid-phase synthesis of triphosphate and diphosphate ONs using a DNA/RNA synthesizer.

(Fig. 1). After completion of the syntheses, the products were manually released from the solid support and deblocked employing a standard two-step treatment involving concentrated ammonia/ethanol followed by tetra-*n*-butylammonium fluoride (TBAF, 1 M in THF), both performed at ambient temperature (Fig. 2). The crude products were then purified and desalted using standard procedures (i.e. anion-exchange (IEX) and size exclusion HPLC), providing the pure target RNAs **13**–**16** in good yields (Table 1). Importantly, there were no significant differences in the isolated yields obtained for the triphosphate (**15**–**16**) and non-triphosphate (**13**–**14**) RNAs (Table 1). We then synthesized, in parallel fashion, four different triphosphate RNAs of different length and sequence (**17**–**20**), using three different scales of solid-support (1, 4 and 10 μmol). Similar percent yields were obtained in all cases (Table 1), indicating the scalability of the synthetic method and the lack of sequence dependence.

2.4. Automated parallel synthesis of 5′-diphosphate RNAs

We also successfully extended the automated triphosphate synthesis procedure to the parallel solid-phase synthesis of 5′-diphosphate RNAs. Diphosphate RNAs are important precursors in the enzymatic synthesis of viral or host m^{7}G-ppp mRNAs,[41] as substrates of guanylyl transferases[42] that catalyze the formation of the unique 5′-5′ triphosphate cap linkage. Recently, 5′-diphosphate RNAs have been reported as potential mRNA gene silencing agents

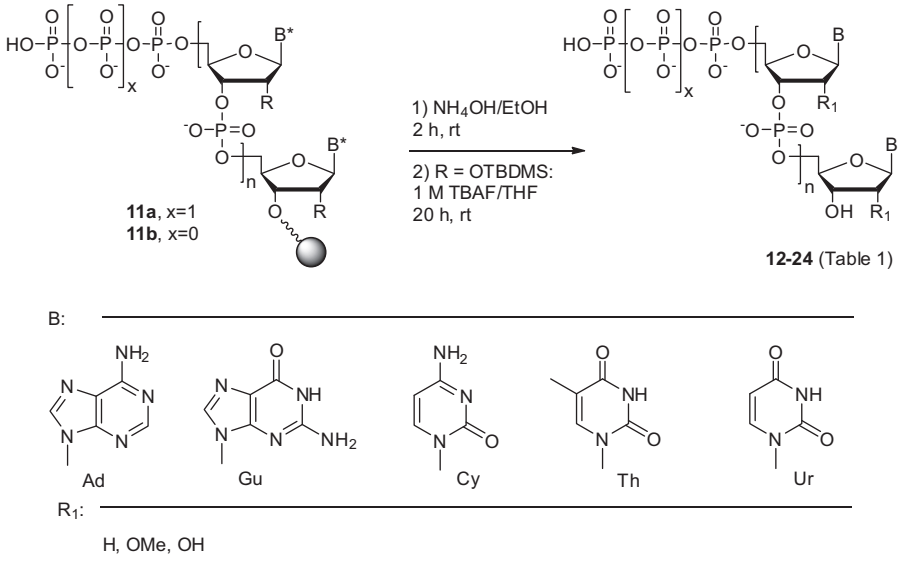

Figure 2. Release and deblock of triphosphate and diphosphate ONs.

Table 1
Data for the automated solid-phase synthesis of triphosphate and diphosphate oligonucleotides

No.	ON 5′-sequence-3′[a]	Length (nt)	Scale (μmol)	Yield[b] (%)
12	**ppp**$(dT)_{10}$[d]	10	1	81[c]
12 A1-A12	**ppp**$(dT)_{10}$[e]	10	1	76[c]
13	AGUAGAAACAAGG	13	1	38
14	AAGAACAUAGGAG	13	1	34
15	**ppp**AGUAGAAACAAGG	13	1	36
16	**ppp**AAGAACAUAGGAG	13	1	33
17	**ppp**AGUAGAAACAAGGGUGUUU	19	1	23
18	**ppp**GUGUAACACGUCUAUACGCCCAGUG	25	4	16
19	**ppp**AGUAGAAACAAGGGUGUUUUUUAUU	25	4	12
20	**ppp**AGUAGAAACAAGGGUGUUUUUU	22	10	25
21	**pp**AGUAGAAACAAGG	13	1	38
22	**pp**AAGAACAUAGGAG	13	1	29
23	**ppp**cuuAcGcuGAGuAcuucGAdTsdT	21	40	22
24	**ppp**AccGAAAGGucuuAccGGAdTsdT	21	40	19

[a] **ppp** = 5′-triphosphate; **pp** = 5′-diphosphate; d = 2′-deoxy; upper case nucleotides = 2′-OH; lower case nucleotides = 2′-*O*-methyl; s = phosphorothioate.
[b] Isolated after IEX-HPLC purification and desalting and calculated based on the measured ODU_{260}.
[c] Calculated from the IEX-HPLC 260 nm UV trace of the crude material based on the integration of the main peak.
[d] Synthesized on ABI-394.
[e] Synthesized on MerMade-192.

or primers and probes in diagnostic applications.[51] Diphosphate RNAs are not commercially available and cannot be readily synthesized using the current chemistries for solid-phase phosphorylation of ONs. Previously, 5′-diphosphate RNAs were only obtained as hydrolysis products.[43] Using our automated procedure (Fig. 1), the simple replacement of the organic salt of pyrophosphate (reagent **8a**) by its phosphate counterpart (**8b**)[44,45] in the final step of the automated synthesis, readily provided the corresponding CPG-supported diphosphate ON (**11b**). The automated two-column parallel synthesis of diphosphate RNAs **21** and **22** was efficiently performed using a procedure identical to that described for **15** and **16** with the phosphate reagent (**8b**) to provide the target diphosphates with isolated yields similar to those obtained for both the RNAs and the triphosphate RNAs (Table 1).

2.5. Automated scale-up parallel synthesis of chemically modified 5′-triphosphate RNAs

Finally, we scaled up the automated synthesis to 40 μmol, and synthesized RNA molecules that contain the standard siRNA-type chemical modifications (i.e., 2′-O-methyl, phosphorothioate, double dT 3′-overhang).[26] The synthetic procedure was modified by increasing the delivery times for each reagent (**5**–**8**), and the waiting times were unchanged, allowing the synthesis to proceed in approximately the same overall time as the smaller scale synthesis (Supplementary Table S3). The two triphosphate RNAs (**23**–**24**) were obtained on the 40 μmol scale in good yields (Table 1) and excellent purity after purification, as seen from the compilation of their physico-chemical characterization profiles (Fig. 5, right panels). The presence of the 5′-triphosphate group was unambiguously confirmed by its unique ^{31}P NMR feature including three resonances at around −6, −8, and −19 ppm corresponding to the γ, α, and β phosphorus atoms, respectively (Fig. 5, center panels). Indirect confirmation for the presence of the triphosphate was also provided by the comparison of the MALDI mass spectra (MS) between the triphosphorylated and non-triphosphorylated RNAs (Fig. 5, left panels).

2.6. Preparation of chemically modified 5′-triphosphate siRNA

The efficient chemical synthesis of modified triphosphate RNAs allowed us to successfully prepare 5′-triphosphate siRNA

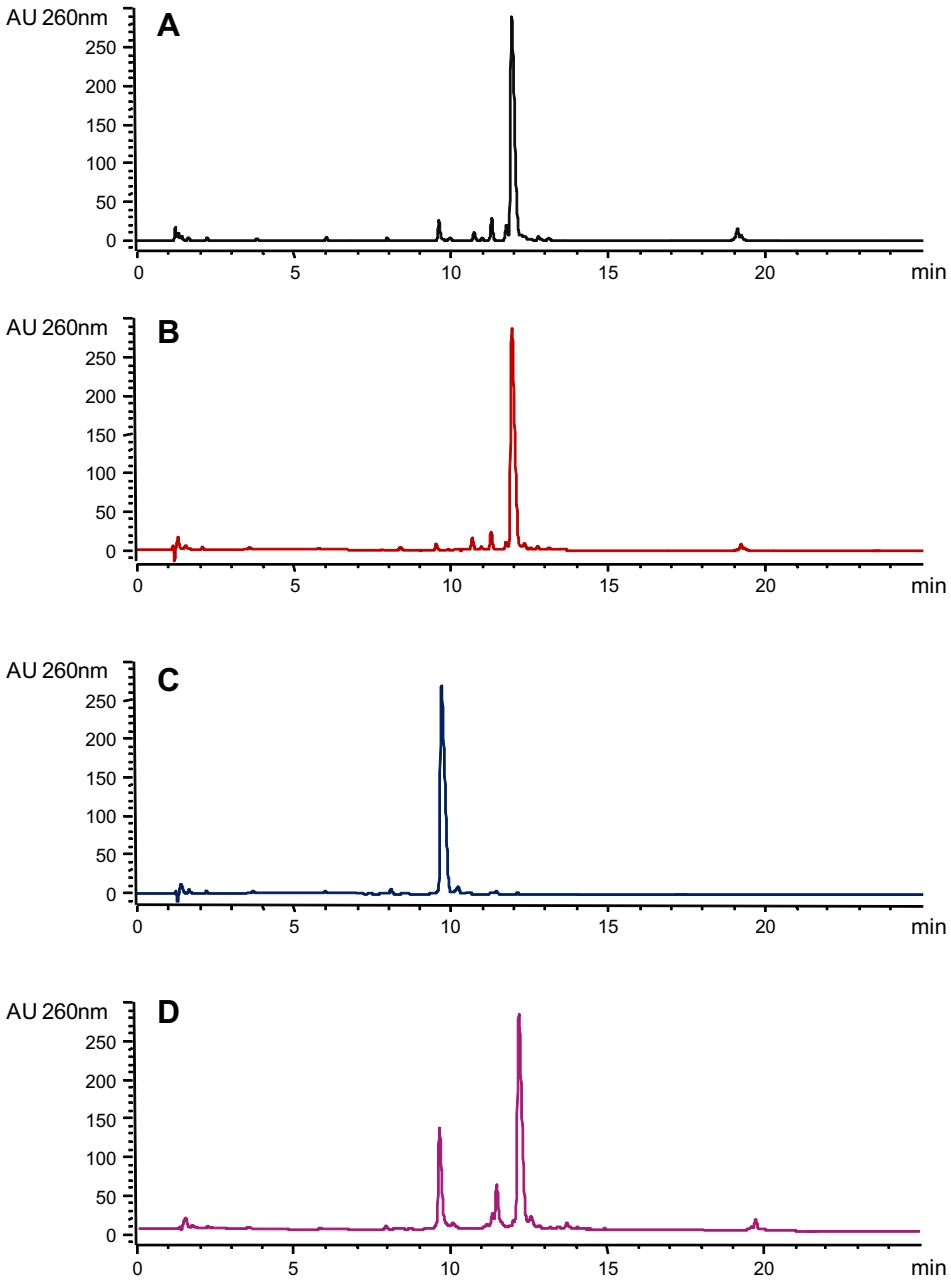

Figure 3. Anion-exchange HPLC profiles of: (A) crude ppp-$(dT)_{10}$ prepared according to the previously published manual procedure[29] using CCl_4; (B) crude ppp-$(dT)_{10}$ **12** prepared following the automated procedure using $CBrCl_3$ depicted on Figure 1; (C) crude $(dT)_{10}$; (D) crude ppp-$(dT)_{10}$ prepared following an automated version of the procedure using 2-chloro-1,3,2-benzodioxaphosphorin-4-one (see text). The crude samples were injected in HPLC after cleavage of the material from the solid support. 260 nm UV spectra were normalized to the height of the highest peaks.

(ppp-siRNA) duplexes, containing 5′-triphosphorylated passenger (sense) strands. The siRNAs were designed to target a portion of the firefly luciferase (Luc) gene, expressed by a dual Renilla/firefly Luc reporter plasmid in HeLa cells. The sequence was that of a previously used potent Luc-targeting siRNA duplex. Both the non-triphosphorylated (duplex **34**) and the 5′-triphosphate counterpart (ppp-siRNA duplex **33**) (Table 2) were prepared. In order to fully distinguish between an RNAi-mediated and any potential immune response-stimulated Luc suppression, we designed another set of triphosphate and non-triphosphate siRNAs (duplexes **31** and **32**) using a sequence that does not affect Luc expression as the negative control (Table 2). The synthetic chemically modified 5′-triphosphate RNA single passenger strands (Table 1, **23** and **24**) were synthesized using our automated method and used for the preparation of the corresponding ppp-siRNAs duplexes.

A chemically synthesized siRNA duplex is usually prepared by annealing of an equimolar mixture of the sense and antisense single RNA strands by heating the mixture to 95 °C followed by slow cooling to ambient temperature. Importantly, as the 5′-triphosphate group is a phosphoric anhydride, it is unstable to acidic conditions and heating[30]—resulting in the hydrolytic cleavage between the γ–β and β–α phosphates. Thus, the thermal annealing of ppp-siRNAs represented a critical step. In order to determine the optimal annealing procedure, we evaluated three different annealing conditions for the preparation of ppp-siRNA duplexes **31** and **33**: (1) thermal annealing in water; (2) thermal annealing in

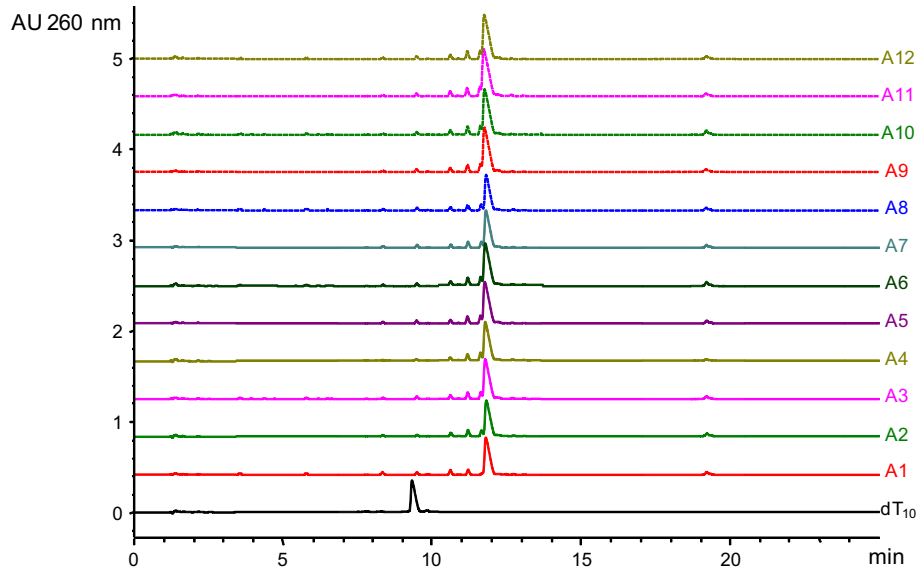

Figure 4. Overlay of IEX-HPLC profiles of crude $(dT)_{10}$ and twelve crude ppp-$(dT)_{10}$ (**12 A1–A12**) synthesized in row A (A1–A12) of the 96-well plate using a MerMade-192 high-throughput synthesizer. The crude samples were injected in HPLC after cleavage of the material from the solid support.

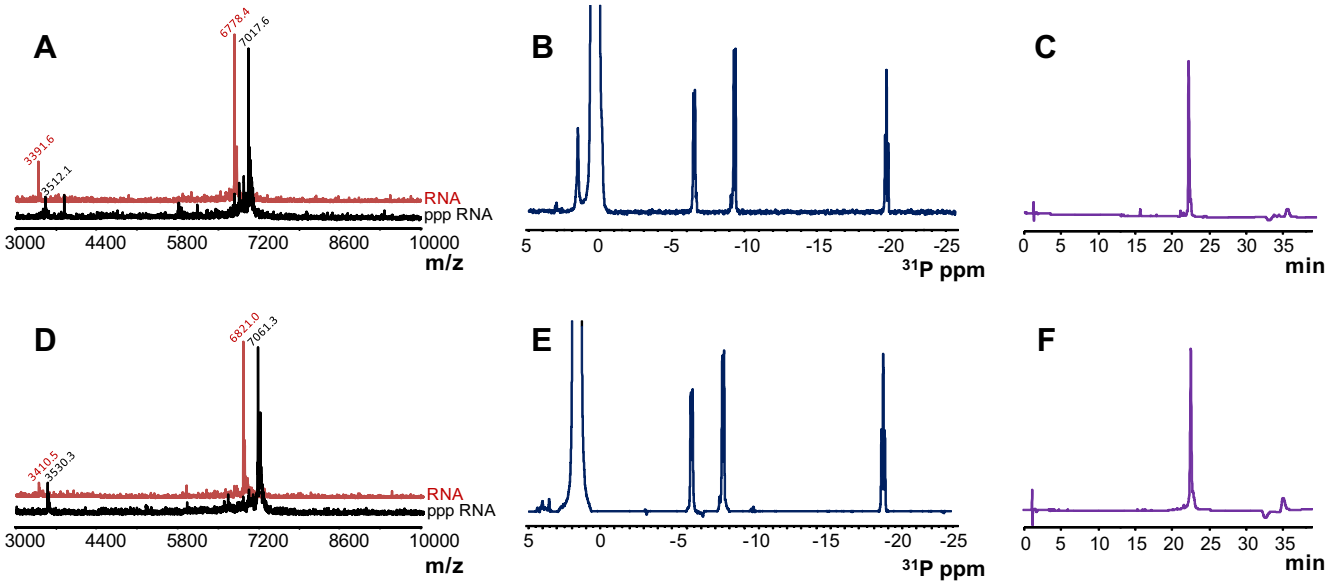

Figure 5. Physico-chemical characterization profiles for compounds **23** and **24**. (A, D) (left panels): MALDI-TOF MS spectra. The spectra in red represent the respective non-triphosphate RNAs **27** and **28**, demonstrating the absence of the triphosphate group (−240 Da); (B, E) (middle panels): ^{31}P NMR spectra in D_2O (referenced to external H_3PO_4) and (C, F) (right panels): IEX-HPLC profiles under denaturing conditions of compounds **23** and **24**, respectively (see Section 5 for details).

Table 2
List of chemically synthesized siRNA and ppp-siRNA duplexes

Duplex no.	ON no.	Strand (ss/as)[a]	ON 5′-sequence-3′[b]
31	**23**	ss	**ppp**cuuAcGcuGAGuAcuucGAdTsdT
	25	as	UCGAAGuACUcAGCGuAAGdTsdT
32	**27**	ss	cuuAcGcuGAGuAcuucGAdTsdT
	25	as	UCGAAGuACUcAGCGuAAGdTsdT
33	**24**	ss	**ppp**AccGAAAGGucuuAccGGAdTsdT
	26	as	UCCGGuAAGACCUUUCGGUdTsdT
34	**28**	ss	AccGAAAGGucuuAccGGAdTsdT
	26	as	UCCGGuAAGACCUUUCGGUdTsdT

[a] ss = sense (passenger) strand; as = antisense (guide) strand.
[b] **ppp** = 5′-triphosphate; d = 2′-deoxy; upper case nucleotides = 2′-OH; lower case nucleotides = 2′-*O*-methyl; s = phosphorothioate.

phosphate buffered saline (PBS) and (3) non-thermal annealing by lyophilization from water. Although we did not observe any major degradation of the triphosphate group in any of the annealing conditions evaluated (Fig. 6), the thermal annealing in water resulted in partial triphosphate decomposition (ca. 20%), whereas the annealing at room temperature by lyophilization produced ppp-siRNA duplexes with no noticeable degradation of the triphosphate moiety, confirmed by LC/MS analysis (Supplementary Fig. S1). The thermal annealing in PBS buffer showed less hydrolysis of the triphosphate than that in water (Supplementary Fig. S1). The room temperature annealing by lyophilization from water was therefore selected as the optimal annealing method, efficiently providing the target ppp-siRNA duplexes **31** and **33** (Fig. 6).

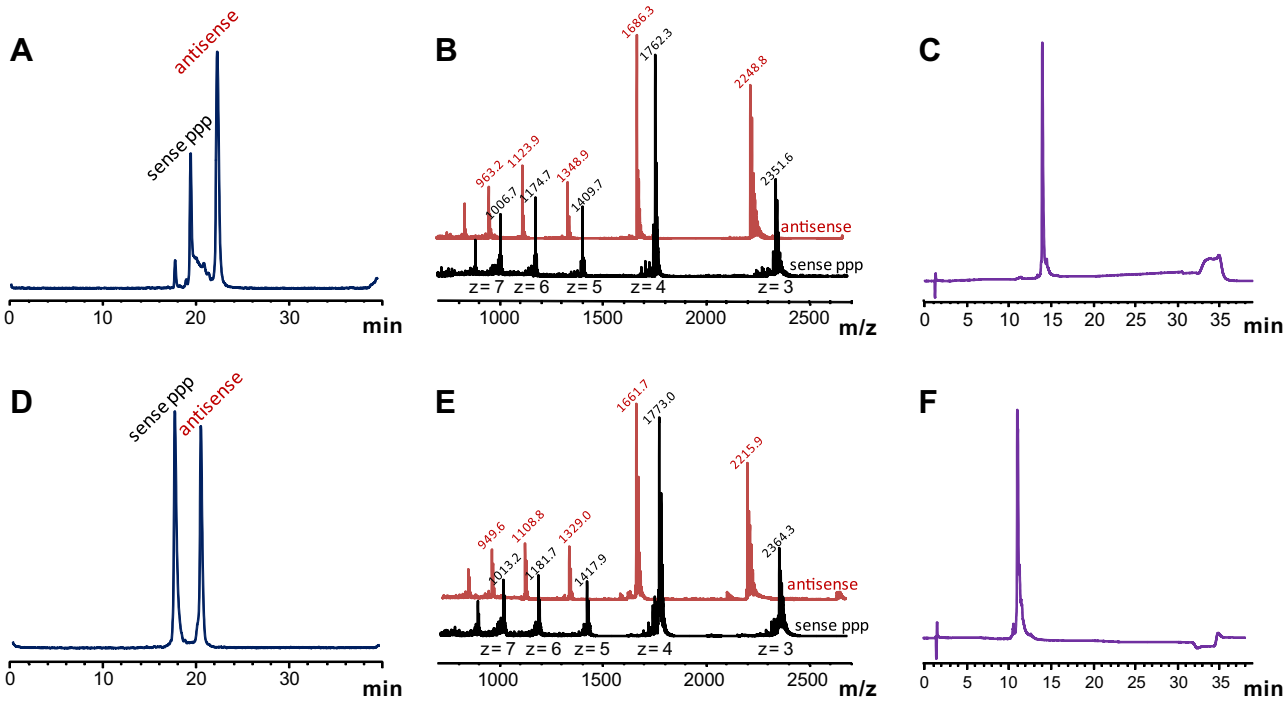

Figure 6. Physico-chemical characterization profiles for ppp-siRNA duplexes **31** and **33**. (A, D) (left panels): RP-HPLC spectra using denaturing conditions demonstrating the presence of the two strands; (B, E) (middle panels): overlay of the LC/MS mass spectra for each one of the strands of the RP-HPLC spectra. The traces in black correspond to the MS of the ppp-sense strands and the traces in red to the MS of the antisense strands; (C, F) (right panels): IEX-HPLC spectra using non-denaturing conditions. The observed single peak corresponds to the existence of a discrete duplex structure.

2.7. RNAi silencing activity of 5′-triphosphate siRNAs

The siRNA duplexes **31**–**34** were evaluated for their RNAi-mediated silencing of the firefly luciferase gene. The siRNAs were transfected into HeLa cells stably expressing the reporter genes with Lipofectamine RNAi-Max. After 24 h, the non-ppp-siRNA control (**32**) inhibited luciferase expression with an IC_{50} of 100 pM (Fig. 7). The corresponding ppp-siRNA (**31**) was active at a similar level, indicating that the presence of a 5′-triphosphate motif did not induce any adventitious Luc suppression deriving from immune response stimulation (i.e., IFN triggering following RIG-I activation, see Section 3) and also that the triphosphate did not inhibit the silencing activity. These observations were further confirmed by the data obtained for the duplexes ppp-siRNA (**33**) and siRNA (**34**). Similar levels of gene silencing (IC_{50} = 10 pM) were observed for both of these optimally designed, potent siRNAs. As was seen for the non-potent set of siRNAs, the siRNA with a 5′-triphosphorylated sense strand (**33**) did not show any significant gain or loss of gene silencing levels and Luc expression as compared to the corresponding non-ppp-siRNA **34** (Fig. 7).

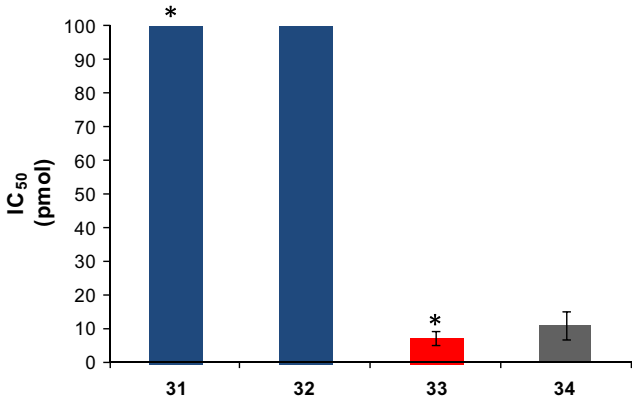

Figure 7. IC_{50} values of RNAi-mediated luciferase gene silencing in HeLa cells for compounds **31**–**34**. The data represents average values from two repeated duplicate experiments. siRNAs (x axis): blue—non-potent chemically modified ppp-siRNA (**31**) and non-ppp-siRNA (**32**); red—potent chemically modified ppp-siRNA (**33**); grey—non-ppp chemically modified potent siRNA (**34**). * = ppp-siRNA.

2.8. Immunostimulatory profiles of 5′-triphosphate siRNAs in human whole blood and in vitro activation of RIG-I

The siRNA duplexes **31**–**34** were further evaluated for their global immunostimulatory profiles in human whole blood using an immunoassay kit designed to detect a variety of human cytokines, chemokines, and growth factors. The siRNAs were transfected using the DOTAP liposomal transfection reagent. No significant immunostimulation was detected for the non-ppp-siRNAs (**32** and **34**), as the levels of detected immune response remained comparable to the levels of the negative controls (Fig. 8A). Slightly elevated immunostimulatory profiles were observed for the ppp-siRNAs (**31** and **33**, Fig. 8A); however levels were relatively weak, similar to those of the negative controls.

Additionally, the chemically modified ppp-siRNAs (**31** and **33**) did not demonstrate any strong specific 5′-triphosphate dependant in vitro RIG-I activation in murine B16-Blue type-I interferon (IFN) sensor cells (Fig. 8B). The siRNA duplexes were first transfected into the murine cells using the LyoVec transfecting reagent. Post incubation, the supernatants were separated and then incubated with the Quanti-Blue reagent. Upon reaction with Quanti-Blue, the levels of secreted embryonic alkaline phosphatase (SEAP), resulting from the in vitro activation of RIG-I, were quantified by photo spectrometry at the wavelength of 655 nm. No significant difference between the non-ppp-siRNAs (**32** and **34**) and the ppp-siRNAs (**31** and **33**) was observed (Fig. 8B), indicating the lack

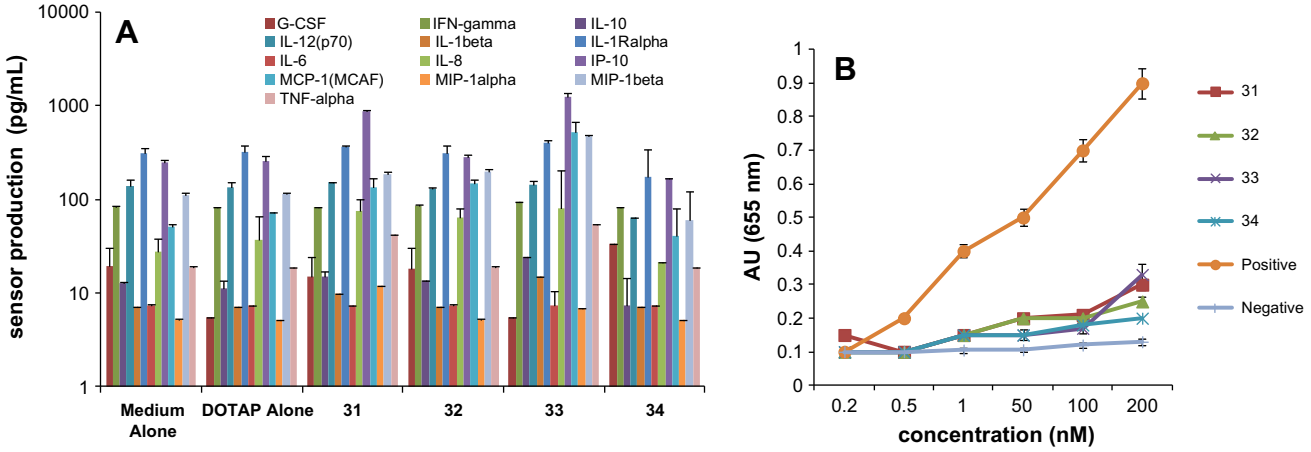

Figure 8. (A): Global immunostimulatory profiles in human whole blood for compounds **31**–**34**. The data represents average values from two repeated duplicate experiments using whole blood from two independent donors. The various sensors measured are labeled in the legend. (B): Expression of IFN-αβ activities determined by the measurement of secreted embryonic alkaline phosphatase (SEAP) resulting from the activation of RIG-I in murine B16-Blue IFN sensor cells for compounds **31**–**34**. The sequences of the positive and negative control dsRNAs are given in the Materials and Methods section. The data represents average values from two repeated duplicate experiments.

of a strong in vitro RIG-I activation generated by the presence of a 5′-triphosphate group in of these chemically modified siRNAs (**31** and **33**).

3. Discussion

The major role of synthetic biophosphate analogues as essential research and therapeutic tools prompted us to develop an automated method for their synthesis as an improved alternative to their enzyme-mediated biocatalytical preparation. The efficient solid-phase synthesis of 5′-polyphosphate nucleosides and oligonucleotides has long been limited due to the lack of robust universal chemical procedures and the failure to enable their automated high-throughput and combinatorial synthesis.[30–32] To our knowledge, the reported herein procedure is the first-to-date automated solid-phase triphosphate oligonucleotide synthesis. It successfully employs the common iterative synthesis, deblock, and isolation steps in standard oligonucleotide synthesis (Fig. 1).[27] A variety of unique unmodified and chemically modified 5′-polyphosphate oligonucleotides were successfully synthesized in good isolated yields. We applied the automated synthetic approach to oligonucleotides of different length, composition, and nature of the 5′-terminal nucleotide, demonstrating the universality of the method. Importantly, we did not observe any notable difference between the isolated yields of recovered triphosphate and non-triphosphate RNAs (Table 1). Moreover, the synthesis involved the use of commercially available, safe, stable, and inexpensive reagents, particularly advantageous and attractive for their use in automated solid-phase synthesis.

We compared our method to an automated version of the current state-of-the art solid-phase 5′-triphosphate oligonucleotide synthesis method, employing the 2-chloro-1,3,2-benzodioxaphosphorin-4-one reagent.[31,32] In addition to difficulties in using this reagent with a DNA/RNA synthesizer (see Section 2.1), the automated synthesis efficiency with the 2-chloro-1,3,2-benzodioxaphosphorin-4-one reagent was clearly inferior to that obtained using our automated procedure (Fig. 3B vs Fig. 3D). We were able to execute the triphosphate synthesis method in a 96-well plate format (Fig. 4). As expected, the high-throughput synthesis presents a great advantage, as the single-column 1 μmol synthesis of one triphosphate oligonucleotide required 10.9 h, while the parallel 12-well 1 μmol synthesis of twelve triphosphate oligonucleotides required only 16.7 h (see Supplementary data for reaction times). The method was also successfully scaled-up and applied for syntheses on 1, 4, 10, and 40 μmol scales.

In 2008, primary work by Poeck et al.[20] described the potential therapeutic use of bi-functional ppp-siRNAs in vivo as a single molecule-based synergy approach in which RIG-I activation corrects the immune response and in which RNAi-mediated gene silencing down-regulates proteins involved in key molecular events that govern tumor cell survival.[20] As the recognition of 5′-triphosphate RNA by RIG-I is known to be independent of the RNA sequence[8] and RNAi-induced gene silencing may not be drastically inhibited by the presence of a 5′-triphosphate, both biological activities can theoretically be effected by a unique ppp-siRNA molecule.[20,46] Initial studies[20] used only in vitro transcribed, chemically unmodified ppp-siRNAs, which are not suitable for therapeutic development, and are known to activate the immune system independently of the presence of a 5′-triphosphate.[16–18,47] Fortunately, immune response stimulation by synthetic siRNAs can be completely abrogated by the incorporation of different chemical modifications (e.g., several 2′-*O*-methyl nucleotides) into one or both strands of the siRNA duplex,[48,49] without hampering its gene silencing activity. Therefore, one should be able to design siRNAs that would be potentially immunosilent to all sensors except RIG-I by the introduction, at selected positions, of 2′-*O*-methyl chemistry into the siRNA duplex, along with the incorporation of the triphosphate group as the RIG-I key recognition motif at the 5′ end of the passenger (sense) strand. Such representative chemically modified 5′-triphosphate siRNA (ppp-siRNA) duplexes, containing 5′-triphosphorylated passenger strands were designed and synthesized in this study (Table 2). Conditions for duplex annealing that did not compromise the stability of the fragile 5′-triphosphate moiety were established.

The initial results of the biological evaluation of two ppp-siRNAs demonstrated that the addition of a triphosphate group to the 5′-end of the passenger strand in two different sets of chemically modified siRNAs did not alter their intrinsic gene silencing properties. Under the transfection conditions employed, ppp-siRNAs and non-ppp-siRNAs showed similar silencing of the firefly luciferase target in HeLa cells (IC_{50} = 10 pM, Fig. 7). While confirming the hypothesis that the addition of a 5′-triphosphate group to a synthetic siRNA does not inhibit its gene silencing efficacy, the in vitro silencing data from the two ppp-siRNAs did not show

additional efficacy resulting from the presence of the triphosphate groups. The conservation of the in vitro RNAi activity of 5′-triphosphate siRNAs is important for the establishment of potent synthetic bi-functional ppp-siRNAs.[20]

The ppp-siRNAs did have slightly elevated immunostimulatory profiles in human whole blood relative to the non-phosphorylated siRNAs. When compared to the levels of cytokine activation by the negative controls and the non-ppp-siRNAs (Fig. 8A), the target ppp-siRNAs showed slightly elevated immunostimulation profiles, although still globally remaining at relatively weak levels of immunostimulation. Furthermore, we were unable to detect strong specific in vitro RIG-I activation conferred by the presence of the 5′-triphosphate group in the two ppp-siRNAs.[8] Indeed, after transfection of the siRNAs in IFN-sensor cells, only the positive 5′-triphosphate dsRNA control (see Section 5.8) produced a strong positive response in the RIG-I assay (Fig. 8B). As both sets of chemically modified ppp-siRNAs and non-ppp-siRNAs demonstrated similar low levels of RIG-I activation (Fig. 8B), the ppp-siRNAs do not seem to be efficient ligands for RIG-I.[19,20,24] Poor binding to RIG-I by these chemically modified ppp-siRNAs could be due to the extensive introduction of 2′-*O*-methyl chemical modifications within the siRNA duplex[24] or to the non-blunt-end nature of the duplexes.[19] Importantly, the unmodified in vitro transcribed ppp-siRNAs used in the study by Poeck et al.[20] are not likely a useful therapeutic format. We expect that future preclinical and clinical development of ppp-siRNAs will be based on chemically modified siRNA architectures,[13–15] to which the 5′-triphosphate group would be added. However, it appears clear that a large screen of different novel ppp-siRNA designs needs to be performed in order to successfully identify potent bi-functional ppp-siRNAs.

4. Conclusion

The reported fully automated triphosphate and diphosphate synthesis represents a significant progress towards the establishment of modern solid-phase parallel and high-throughput synthesis methods for 5′-triphosphate nucleosides and oligonucleotides. We expect that the automated synthetic approaches described in this work will enable the screening of a large number of optimized ppp-siRNA therapeutic candidates, which taken together with the future establishment of specific in vitro and in vivo evaluation models will fully reveal the therapeutic potential of this novel class of molecules in future antitumor and antiviral therapies. Independent of the utility of these designs in siRNA-based therapeutics, the synthesis method described herein is robust and applicable to all families of nucleosides and oligonucleotides and will prove useful for the synthesis of their 5′-polyphosphorylated analogues and their various applications in diagnostics or mechanistic studies.

5. Experimental

5.1. Preparation of reagents (5–8) for the automated synthesis of 5′-triphosphate and 5′-diphosphate oligonucleotides

All chemical reagents and solvents were used as received, unless otherwise stated. Reagent **5**:2 mL of diphenyl phosphite was mixed with 8 mL of anhydrous pyridine in an amber glass bottle. Reagent **6**:1 mL of 1 M aqueous triethylammonium bicarbonate was mixed with 5 mL of sterile nuclease-free water and 4 mL of acetonitrile in an amber glass bottle. Reagent **7**: 600 mg of imidazole was dissolved in a mixture of 2 mL of N,O-bis(trimethylsilyl)acetamide (BSA), 4 mL of anhydrous acetonitrile, 4 mL of bromotrichloromethane and 0.4 mL of triethylamine. The mixture turned dark yellow-brown after the addition of triethylamine. The solution was dried and stored over 4 Å activated molecular sieves in an amber glass bottle. Reagent **8a**: 4 g of tributylammonium pyrophosphate was dissolved in a mixture of 5 mL of anhydrous DMF and 5 mL of anhydrous acetonitrile. The solution was dried and stored over 4 Å activated molecular sieves in an amber glass bottle. Reagent **8b**: 2 g of tributylammonium phosphate was dissolved in a mixture of 5 mL of anhydrous DMF and 5 mL of anhydrous acetonitrile. The solution was dried and stored over 4 Å activated molecular sieves in an amber glass bottle. Tributylammonium phosphate is not commercially available and was prepared with modifications of previously published procedures[44,45,50]. First, 0.71 g (5 mmol) of sodium phosphate dibasic were dissolved in 50 mL of sterile nuclease-free water and eluted through a glass column filled with 20 mL of wet DOWEX-50WX8 resin, H^+ form. After ion-exchange passage through the column, the solution was collected in a 250-mL flask, containing 20 mL of absolute ethanol and 2.5 mL of tributylamine, while stirring at 0 °C. The column was rinsed with 50 mL of water (outlet pH 5 to 6). The solvents were evaporated, and the residue was coevaporated four times with absolute ethanol. The residue was lyophilized from water, then from water/dioxane (three times). Tributylammonium phosphate was obtained as a white hygroscopic powder (1.1 g, 80%). Reagents (**5**–**8**), prepared as described above, were stable for at least one week on the oligonucleotide synthesizer, and several weeks at 4 °C, as they were successfully re-used for synthesis with no significant deterioration of quality and corresponding yields. The average volume of each reagent (**5**–**8**) required for single triphosphate/diphosphate incorporation on an ABI-394 was of 2 mL for the 1–10 μmol scale cycle and of 5 mL for the 40 μmol scale cycle. The average volume of each reagent required for twelve triphosphate incorporations (one 12-well row of a 96-well plate) on a MerMade-192 was 20 mL for the 1 μmol scale cycle.

5.2. Synthesis of 5′-triphosphate and 5′-diphosphate oligonucleotides on ABI-394 synthesizer

Oligonucleotides were synthesized using an ABI-394 DNA/RNA synthesizer. Sterling solvents/reagents and UltraMild solvents/reagents for ABI synthesizer; DNA, RNA and UltraMild RNA 500 Å CPG solid-supports; DNA, RNA, 2′-O-Me RNA, UltraMild RNA and UltraMild 2'-O-Me RNA phosphoramidites were all purchased from Glen Research and used as received. Low-water acetonitrile was purchased from EMD Chemicals. DNA and RNA oligonucleotides were synthesized using modified synthesis cycles, based on those provided with the instrument (see Supplementary data). Three different synthesis cycles were used for the synthesis scales of 1 - 10 and 40 μmol, respectively (Supplementary Table S2). Phosphoramidite solutions, 0.1 M in anhydrous acetonitrile, were used, except for 2′-*O*-methyluridine, for which 0.1 M solution in acetonitrile/THF—1:1 (v/v) was used. A solution of 5-ethylthio-1-*H*-tetrazole, 0.25 M in anhydrous acetonitrile was used as the activator. The triphosphate/diphosphate synthesis cycle was entered as the 'end procedure', and used a custom-designed synthesis cycle, enabling the delivery of the in-house prepared reagents **5**–**8**. Two different synthesis cycles were used for the synthesis scales of **1**–**10** and 40 μmol, respectively (Supplementary Table S3). Detailed synthesis cycles and procedures are given in the Supplementary data. After completion of the automated synthesis, the solid support was washed with anhydrous acetonitrile and dried with argon. It was then manually deblocked using a mixture of 30%-NH_4OH/absolute ethanol—3:1 (v/v, 1 mL/μmol of solid support) for 2 h at room temperature, filtered off, rinsed with sterile water and the filtrates were freeze-dried. The dry residue was treated with 1 M TBAF in THF (1 mL/μmol of solid support) for 20 h at room temperature. The crude oligonucleotides were purified by

anion-exchange HPLC on an AKTA Purifier-100 chromatography system using a DNAPac PA-100 semi-preparative column (22 × 250 mm) or a AP-5 glass column (50 × 300 mm) custom-packed with the DNATSK-Gel Super Q-5PW support (TOSOH Bioscience), using a linear gradient of 0.22 M–0.42 M NaBr in 0.02 M phosphate buffer/10% (v) acetonitrile, pH 8.5 in 120–150 min, at room temperature. All single strands were purified to >85% HPLC (260 nm) purity and then desalted by size exclusion chromatography on an AKTA Prime chromatography system using an AP-2 glass column (20 × 300 mm) custom-packed with Sephadex G25 (GE Healthcare), eluted with sterile nuclease-free water. The isolated yields for the final oligonucleotides were calculated based on the respective ratios of measured/theoretical 260 nm optical density units, and their purity and identity were determined by analytical IEX-HPLC and LC/MS (Table S1).

5.3. High-throughput synthesis of 5′-triphosphate oligonucleotides on MerMade-192 synthesizer

Oligonucleotide synthesis was performed on a 1 μmol scale in a 96-well plate on a MerMade-192 synthesizer. Empty synthesis plates, empty 1 μmol synthesis columns and empty bottles were purchased from BioAutomation. DNA 500 Å T-CPG solid-support, DNA T-phosphoramidite and Sterling solvents/reagents for MerMade synthesizer were purchased from Glen Research. Phosphoramidite solution, 0.1 M in anhydrous acetonitrile was used, and a 0.6 M solution of 5-ethylthio-1-*H*-tetrazole in anhydrous acetonitrile was used as the activator. The triphosphate synthesis cycle was entered as **4** additional steps in the custom-designed synthesis cycle, enabling the delivery of in-house prepared reagents **5**–**8**. Detailed synthesis cycles and procedures are given in the Supplementary data. After completion of the automated synthesis, the solid support was washed with anhydrous acetonitrile and dried with argon. It was then manually deblocked using a mixture of 30% NH_4OH/absolute ethanol—3:1 (v/v, 1 mL/well) for 2 h at room temperature in the 96-well cleavage apparatus. The crude deprotected triphosphate oligonucleotides were analyzed by IEX-HPLC (Fig. 3 and Supplementary data).

5.4. Analytical methods

LC/ESI-MS was performed on an Agilent 6130 single quadrupole LC/MS system using an XBridge C8 column (2.1 × 50 mm, 2.5 μm) at 60 °C. Buffer A consisted of 100 mM 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP)/16.3 mM triethylamine (TEA) in H_2O and buffer B was 100% methanol. A gradient from 2% to 29% of buffer B over 26.8 min with flow rate of 0.25 mL/min was employed. Analytical IEX-HPLC was performed using a DNAPac PA-200 analytical column (4 × 250 mm). Buffer A consisted of 0.02 M Tris–HCl, 1 mM EDTA/10% (v) acetonitrile, pH 8.5 and buffer B was buffer A plus 1 M NaBr, pH 8.5. A gradient of 15 to 50% of buffer B over 29 min, at a flow rate of 1.0 mL/min was used. The column temperature was set at 20 °C for the non-denaturing conditions and at 65 °C for the denaturing conditions. MALDI-TOF MS analysis was performed on a PE Voyager Pro instrument. A 4:1 (v/v) mixture of 2,4,6-trihydroxyacetophenone monohydrate (THAP) and ammonium citrate was used as the matrix. Matrix and desalted sample were combined in equal volumes and spotted on the stainless steel plate. The delay time was 150 ns and the acceleration voltage was 20 kV. ^{31}P NMR was recorded on a Varian 400 instrument at the frequency of 162 MHz. The triphosphate oligonucleotide (300 ODU at 260 nm) was dissolved in 0.6 mL of 5 mM EDTA solution in H_2O/D_2O–1:1 (v/v), buffered to pH 7 with 2 M Tris–HCl buffer. The ^{31}P chemical shift was referenced to external H_3PO_4. The relaxation delay parameter (d_1) was set at 0.3 s.

5.5. siRNA and 5′-triphosphate siRNA duplex formation

Hybridization to generate siRNA and ppp-siRNA duplexes was performed using three different annealing conditions: 1) thermal annealing in water—equimolar amounts of purified complementary sense (passenger) and antisense (guide) strands were mixed to a final concentration of 20 μM in sterile nuclease-free water and annealed by heating in a water bath at 95 °C for 5 min and cooling to room temperature over a period of approximately 12 h; 2) thermal annealing in PBS buffer—equimolar amounts of purified complementary strands were mixed to a final concentration of 20 μM in 1 × PBS buffer, pH 7.4 and annealed by heating in a water bath at 95 °C for 5 min and cooling to room temperature over a period of approximately 12 h; 3) non-thermal annealing by lyophilization from water—equimolar amounts of purified complementary strands were mixed to a final concentration of 100 μM in sterile nuclease-free water. The solution was frozen and lyophilized from water over a period of approximately 12 h. The dry material was re-dissolved to a final concentration of 20 μM in 1 × PBS buffer, pH 7.4. In each case, the formation of the duplex structure was confirmed by non-denaturing IEX-HPLC. The results are plotted in the Supplementary data.

5.6. Cell culture, transfection and RNAi activity assay in HeLa cells

Tissue culture medium, trypsin and Lipofectamine RNAi-Max were purchased from Invitrogen. HeLa cells were obtained from the American Type Tissue Collection. The luciferase plasmids, *Renilla* luciferase (pRL-CMV), firefly luciferase (pGL3) and the Dual Glo Luciferase Assay kit were purchased from Promega. HeLa SS6 cells, stably transfected with the luciferase plasmids, *Renilla* luciferase (pRL-CMV) and firefly luciferase (pGL3), were grown at 37 °C, 5% CO_2 in Dulbecco's modified Eagles's medium (DMEM, GIBCO) supplemented with 10% (v) fetal bovine serum (FBS) and 0.5 μg/mL zeocin and 0.5 μg/mL puromycin (selective for cells transfected with plasmids). The cells were maintained in exponential growth phase. For the luciferase assay the cells were plated in 96-well plates (0.1 ml medium per well) to reach ~90% confluence at transfection. The cells were grown for 24 h and the culture medium was changed to OPTIMEM 1 (GIBCO), 0.5 mL per well. Transfection of siRNAs was carried out with Lipofectamine RNAiMax (Invitrogen) as described by the manufacturer for adherent cell lines in the siRNA concentration range of 0.002–8.0 nM. The final volume was 150 μL per well. The cells were harvested 24 h after transfection, and lysed using passive lysis buffer (PLB), 100 μL per well, according to the instructions of the Dual-Luciferase Reporter Assay System (Promega). The luciferase activities of the samples were measured using a Victor FL Luminometer. The volumes used were: 20 μL of sample and 75 μL of each reagent (luciferase assay reagent II and Stop and Glo Reagent, respectively). The inhibitory effects generated by siRNAs were expressed as normalized ratios between the activities of the reporter (firefly) luciferase gene and the control (*Renilla*) luciferase gene relative to the mock-treated controls (no siRNA, but with Lipofectamine RNAiMax). Values represent the mean of duplicates. The potency of the siRNAs was determined by calculating the IC_{50} values from the corresponding dose–response curves using XL-Fit software.

5.7. Whole blood assay

Whole blood from two, anonymous, healthy donors was collected in 10 mL 'green-topped' Sodium Heparin Vacutainer tubes (Becton Dickinson) and diluted 1:1—(v/v) in 0.9% Saline (Baxter). 180 μL/well were plated in 96-well, flat bottom plates. siRNA was transfected at a final concentration of 150 nM or 300 nM using

8 μg/mL DOTAP (Roche Applied Sciences). Following incubation at 37 °C, 5% CO_2 for 24 h, plasma was harvested and stored at −80 °C until cytokine/chemokine analysis could be conducted. Cytokine/chemokine analysis was conducted using a Bio-Plex Pro Human Cytokine Assay (BioRad Laboratories) according to manufacturer's instructions.

5.8. Cell culture, transfection and IFN-αβ triggering in murine type I IFNs sensor cells

B16-Blue INF-αβ cells, zeocin and normocin were purchased from InvivoGen. Penicillin-streptomycin-L-glutamine (100×) and RPMI medium were purchased from Invitrogen. Upon arrival, the cells were handled according to the manufacturer's instructions and were grown at 37 °C, 5% CO_2 in Roswell Park Memorial Institute's medium (RPMI, GIBCO) supplemented with 10% (v) FBS, 50 U/mL penicillin, 50 μg/mL streptomycin, 100 mg/mL normocin and 2 mM L-glutamine. Selective antibiotics—100 μg/mL zeocin were added to the growth media after the cells have been passaged twice, according to the manufacturer's instructions. The cells were maintained in exponential growth phase. For the IFN triggering assay, 180 μL of the suspended cells in the growth media, excepting zeocin, were plated in a 96-well plate. Transfection of siRNAs was carried out with LyoVec (InvivoGen) as described by the manufacturer for adherent cell lines, in the siRNA concentration range of 200–0.2 nM. The final volume was 200 μL per well. 24 h after transfection, the plates were centrifuged and the supernatant (20 μL per well) was transferred into another 96-well plate, containing the Quanti-Blue reagent (200 μL per well) (prepared according to the manufacturer's instructions). The plates were incubated for 6 h at 37 °C. The INF-αβ activities of the samples were measured by quantifying the secreted embryonic alkaline phosphatase (SEAP) levels using a Victor FL Luminometer at the 655 nm wavelength. The immuno effects generated by siRNAs were expressed as normalized ratios between the activities of the reporter SEAP secretion for each siRNA and for the positive and negative dsRNA controls (InvivoGen), relative to the mock-treated controls (no siRNA, but with LyoVec). Values represent the mean of duplicates. Sequences of the controls (InvivoGen): Positive ppp-dsRNA control (5′-pppGCAUGCGACCUCUGUUUGA-3′/3′-CGUACGCUGGAGACAAACU-5′); Negative non-ppp-dsRNA control: (5′-GCAUGCGACCUCUGUUUGA-3′/3′-CGUACGCUGGAGACAAACU-5′). The control dsRNAs were used as received and were handled according to the manufacturer's protocol.

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Supplementary data

Supplementary data (supplementary Tables S1–S5, Supplementary Figure S1, Supplementary Methods and Supplementary Dataset S1) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2012.11.043.

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