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Liquid-Phase RNA Synthesis by Using Alkyl-Chain-Soluble Support

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Abstract: Recent progress in the RNA therapeutics has increased demand for the synthesis of large quantities of oligoribonucleotides. The assembly of RNA oligomers relies mainly on solid-phase approaches. These allow rapid product purification and the ability to drive a target reaction to completion through the use of excess reagents. Despite the known advantages of solid-phase synthesis, some issues in the process remain to be addressed, such as low and limited scale, reagent accessibility, and the use of a very large excess of reagents. Herein, we report a highly efficient and practical method of liquid-phase synthesis of RNA oligomers by using alkyl-chain-soluble support. We demonstrate the utility of the liquid-phase method through 21-mer RNA synthesis on a gram scale.

Keywords: liquid-phase synthesis · oligonucleotides · phosphoramidite chemistry · RNA · soluble support

Introduction

Recent advances in short-interfering RNA, microRNA, and their application to therapeutics has increased demand for the synthesis of large quantities of RNA oligomers and their derivatives for animal and clinical trials.[1] Over the past few decades, much of the widespread utilization of synthetic oligonucleotides has been made possible by solid-phase oligonucleotide synthesis. Indeed, insoluble supports for solid-phase synthesis have been a fundamental technology in automated synthesis and combinatorial chemistry. These allow rapid product purification and the ability to drive a target reaction to completion through the use of excess reagents.[2] However, despite the known advantages of solid-phase synthesis, some issues need to be addressed in the process, such as low and limited scale, reagent accessibility, and the use of a large excess of reagents due to nonequivalent reactivity on the inherently unequal interface of the insoluble resin structure. Furthermore, RNA synthesis has been regarded as far more difficult than DNA synthesis, mainly because of the problems associated with 2′-O-functional groups, such as steric hindrance and phosphodiester backbone cleavage by 3′ to 2′ phosphate migration.[2c,3]

On the other hand, the use of soluble supports provides an alternative platform for high-throughput organic synthesis by incorporating the beneficial aspects of both conventional solution-phase and solid-phase chemistry. By establishing homogeneous reaction conditions while still facilitating product separation, these liquid-phase methodologies have demonstrated utility in a variety of areas including peptide and DNA synthesis, small-molecule organic synthesis, polymer-supported reagents and catalysts.[4,5] Although great efforts have been made to construct homogeneous solutions through the design and improvement of soluble supports, they have often not contributed to enhancement of reagent accessibility. This has resulted in the requirement of longer reaction times or excess reagents. A liquid-phase solution system is microscopically homogeneous and isotopic, but heterogeneous on the molecular scale. This is caused by a wide range of complex aggregations, which support soluble form due to the multiple weak bond interactions of their backbone structure, side-chains, spacers, and the attached molecules. The aggregate heterogeneity provides nonequivalent reactivity and reagent accessibility in a similar manner as solid-phase chemistry, resulting in unpredictable difficulty in the development of scaled-up, cost-effective synthesis. In particular, although in some studies on the liquid-phase approach, particularly the soluble support–substrate strategy, its potential was reported by synthesizing preliminary targets, there are only a few examples that demonstrate its usefulness by achieving the synthesis of a difficult synthetic target.

Herein, a highly efficient and practical liquid-phase method of RNA synthesis by using alkyl-chain-type soluble support is described. The utility of the liquid-phase strategy to describe 1-mer RNA synthesis on gram scale is described. We also report aggregation/dispersion behaviors of the alkyl-chain-soluble support with dynamic light scattering analysis.

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Results and Discussion

A liquid-phase synthetic strategy to construct homogeneous reaction conditions, while at the same time facilitating product separation, involves the incorporation of soluble-support carriers into an organic moiety. Alkyl-chain-soluble support, in particular, hydrocarbon backbone structure, plays an important role in the strategy of a liquid-phase synthesis. Specifically, the incorporation of alky-chain-soluble support enhances the reaction efficacy by facilitating easy separation of products via hydrophobic interaction, by ensuring stability of standard reaction conditions, and by enabling direct access to routine analytical methods without redundant handling. Furthermore, the hydrophobicity of the alkyl-chain-soluble support may be compatible with oligonucleotide synthesis, given that anhydrous conditions are essential for the chain elongation process based on phosphoramidite chemistry. In addition, the dispersion and aggregation behavior associated with the structure of soluble support could be easily controllable and simplified, because unlike polymer resins, it only has one discrete molecular weight.

Based on these considerations, we initially synthesized ASS **1**, and its solubility was tested in various solvents. The results are summarized in Table 1. In all cases, the solubility of ASS **1** was unexpectedly low, even when nonpolar *n*-hexane was used. This low solubility was presumably due to hydrogen-bonding interactions on the primary amide linker, which led to its precipitation. Thus, ASS **2** was prepared to avoid these hydrogen-bond interactions. We found that it displayed high solubility in THF, $CHCl_3$, and CH_2Cl_2 (>100 fold compared to ASS **1**), and no solubility in polar solvents, MeOH, MeCN, and EtCN. These solubility results indicated that ASS **2** had suitable properties for liquid-phase RNA synthesis. The solubility control of ASS **2** by appropriate solvent changes allowed conventional liquid-phase separation techniques, such as liquid–liquid extraction, precipitation, and adsorption. In addition, the dispersion properties of the ASSes were markedly affected by both the solvent, and by slight differences in the structure of attached molecules, including the linker region.

Table 1. Solubility of ASS **1** and **2** in various solvents.

Entry	Solvent	Solubility [mM][a]	
		ASS **1**	ASS **2**
1	CH_2Cl_2	0.8	275
2	toluene	1.0	260
3	$CHCl_3$	4.6	304
4	THF	12.7	228
5	cyclohexane	0.4	23.1
6	*n*-hexane	n.d.[b]	n.d.[b]
7	MeOH	n.d.[b]	n.d.[b]
8	MeCN	n.d.[b]	n.d.[b]

[a] Determined from saturated solution of ASS **1** and **2** at 25 °C.

To understand the influence of ASS-attached molecules, we also prepared ASS **3**–**6** and tested their solubility in CH_2Cl_2. As shown in Table 2, the solubility of soluble supports **2** and **4** were high, whereas soluble supports **1**, **3**, **5**, and **6** containing one or two primary amide bonds showed extremely poor solubility in dichloromethane. Despite the attached molecule (thymine), which includes the amide bond that potentially induces hydrogen bonding, solubility of ASS **2** did not decrease (Table 2, entries 2 and 4). In addition, the solubility was only slightly affected by the direction of the primary amide bond (acceptor/donor) or linker length. It should be noted that intermolecular interactions were induced more strongly in the vicinity of the alkyl chains containing the aromatic ring. ASS **2** is apparently suitable for standard chemical RNA synthesis, such as the phosphoramidite strategy, because nucleotide-chain elongation proceeds in the opposite direction from the alkyl region.

Table 2. Solubility of ASS **1**–**6** in CH_2Cl_2.

Entry	ASS	Solubility [mM][a]
1	1	0.8
2	2	275
3	3	3.9
4	4	163
5	5a	0.3
6	5b	1.1
7	6	2.8

[a] Determined from a saturated solution of each compound at 25 °C.

Based on the structural similarity of the synthesized ASSes, we suspected that highly soluble ASS **2** and **4** are distributed by forming aggregations, even when constructed in the homogenous solution phase. The behavior of the aggregates in dispersed media is an important factor in determining reaction equivalency and reagent accessibility. In addition, dispersed media is involved in subsequent separation methods, that is, cyclohexane could potentially lead to liquid–liquid extraction through the thermomorphic system, whereas CH_2Cl_2 would require precipitation with dilution of polar solvent. In particular, MeCN is widely used in phosphoramidite chemistry of solid- and classical solution-phase DNA and RNA synthesis. Based on these observations, we investigated the effect of media on dispersion properties by using dynamic light scattering (DLS) technique (Figure 1).

Based on DLS analysis data, ASS **3** was observed to form nanoparticles in the range of approximately 2.0–200 nm (Figure 2). It formed small aggregates at the first aggregate stage, followed by growing to a size of 39 nm under solvent conditions (Figure 2a), and to 66 nm in (Figure 2b) with monomodal distributions, respectively, within a few minutes. After 5 h, the aggregates increased to an average particle size of 88 and 180 nm in a) and b), respectively. As was predicted, the growth rate in dispersed media based on CH_2Cl_2 was lower than in that of cHex. ASS **1** was not detected in this analysis (measuring limit ca. 0.8-6.500 nm). These results indicated that ASS is involved in the liquid-phase reaction system to exist in an aggregate form, the stability of which varies according to the dispersed media. As a possible explanation, the aggregate propensity is determined by differences in surface structure. The experimental results of DLS

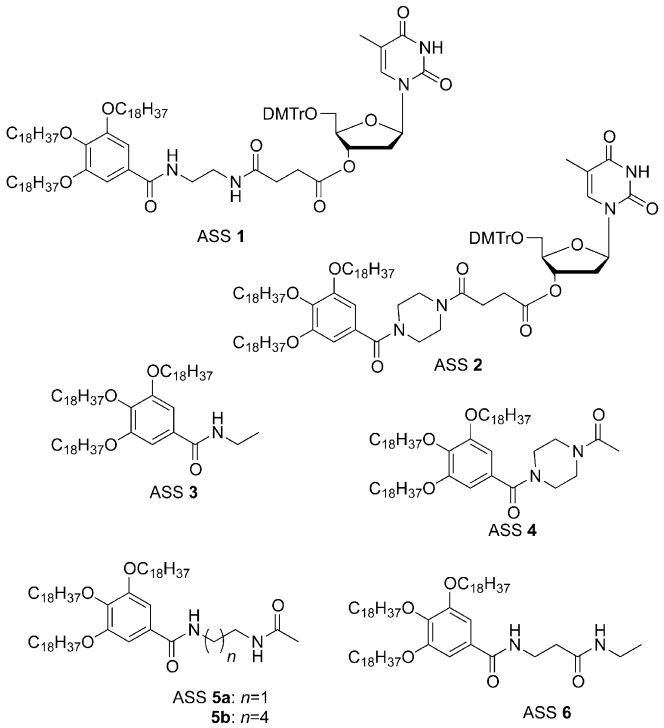


Figure 1. Alkyl-chain-soluble supports.

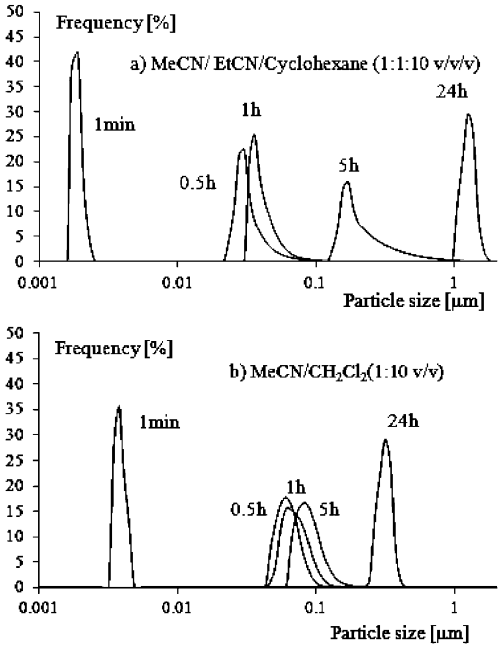


Figure 2. Dispersion analysis of ASS **3** by DLS experiment.

analysis, particularly for the first aggregation size, support this conclusion. Specifically, the instability of the aggregates causes their irreversible growth that provides a reaction environment, in which reactivity of ASS is similar to that of the solid-phase system. In the cases when reactive sites are encapsulated inside aggregation architecture, the reactivity would be decreased markedly. On the other hand, when the aggregation is more stably maintained on the submicron level, ASS reactivity would exhibit that of conventional solution-phase conditions. It was expected that CH_2Cl_2-based media would provide a more relevant and ideal liquid-phase synthetic system. Therefore, the reactivity of ASS was examined by using phosphoramidite coupling reaction.

Reactions were performed in MeCN/CH_2Cl_2 solution (1:10) at room temperature by using 5′-hydroxy free ASS **1a**, **2a**, 5-(benzylmercapto)-1*H*-tetrazole (BMT) as an activator reagent,[6] 5′-Dimethoxytrityl-2′-deoxy-thymidine,3′-[(2-cyanoethyl)-(*N*,*N*-diisopropyl)]-phosphoramidite (dT-phosphoramidite), on a 0.01 M scale. After 15 min, 2-butanone peroxide/CH_2Cl_2 solution was added to the reaction mixture.[7] The mixture was then stirred for 5 min to oxidize the corresponding dinucleoside phosphite to the phosphate. Conversions were determined by ^{1}H NMR analysis. ASS **2a** was allowed to react with an ideal equivalent of phosphoramidite reagents to give product **7** with an excellent conversion rate of 99%. The product was recovered quantitatively by precipitation with methanol.

In a similar manner, the RNA coupling reaction was performed by using rU-phosphoramidite 3′-*O*-(*tert*-butyldimethylsilyl)ribonucleoside 2′-phosphoramidite, which is readily available, although this liquid-phase system was expected to use 2′-*O*-cyanoethyl amidite monomers as a less bulky 2′-hydroxyl protecting group for the improvement of the coupling efficiency.[3] The RNA phosphoramidite reaction proceeded to give the corresponding product **8** in 99% yield. In contrast, the coupling reaction by using ASS **1a** gave unacceptable yield, even though it was conducted by further addition of excess phosphoramidite reagents or longer reaction times. Reactivity of ASS **1a**, which was insoluble in the reaction solvent, appeared to be much lower than that of the insoluble resins used in solid-phase synthesis. This implies that excess aggregation inhibits access to the reagents dispersed in bulk media. Reactivity of ASS **2a**, which was more stably dispersed in CH_2Cl_2/MeCN media, was expected to allow a more reliable liquid-phase RNA synthesis in the homogenous solution.

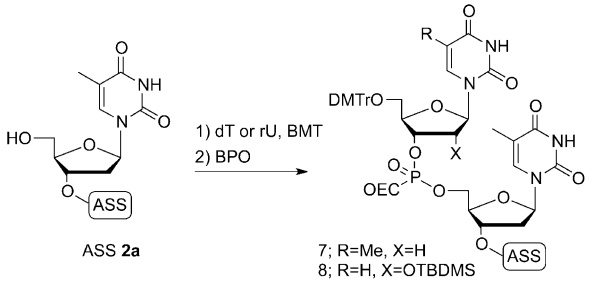
Finally, we examined the synthesis of 5′-r(CUUACGCUGAGUACUUCGA)-d(TT)-3′ as a target RNA oligomer including a TT overhang region.[8] The reaction conditions for all of the steps are summarized in Table 3.[9] As shown in Table 4, acetylation (capping process) and the molecular sieves were not employed herein to simplify the handling and the total reaction and separation steps. Reaction completion was confirmed by monitoring substrate consumption with TLC. Phosphoramidite reagent (2.0 equiv) was required for reaction completion by coupling with 5′-HO-rA moiety in each step. Although the chain elongations did not significantly influence the dispersion of ASS for enhancement of coupling, some small losses occurred during the filtration step in the separation process of 15-21-mer (ca. 10%). The use of hydrophobic adsorption on the C18 silica gel made it possible to effectively recover each of the ASS products. By using this improved recovery manipulation, the target RNA sequences were obtained in good yield

Table 3. Protocol for liquid-phase RNA synthesis.[a]

Elongation process	
phosphoramidite	1.5–2.0 equiv dT, rU, rA(Pac), rG(*i*Pac), rC(Ac)
coupling	BMT (0.25 M) in MeCN/CH_2Cl_2 (1:10)
oxidation	BPO (0.67%)/DMP/CH_2Cl_2[b]
detritylation	DCA (3%) in CH_2Cl_2
capping	–
molecular sieves	–
Cleavage and deprotection process	
cleavage	NH_3 (28%, aq.)/EtOH (3:1)
desilylation	TEA-3HF in NMP
detritylation	TFA (2%) in H_2O

[a] All reactions were performed at RT. [b] Abbreviations: Pac = phenoxyacetyl, *i*Pac = isopropylphenoxyacetyl, Ac = acetyl, BMT = 5-(benzylmercapto)-1*H*-tetrazole, BPO = 2-butanone peroxide, DMP = dimethyl phtalate, DCA = dichloroacetic acid, NMP = *N*-methylpyrrolidone, TBDMS = *tert*-butyldimethylsilyl, TEA = triethylamine, TFA = trifluoroacetic acid, DMTr = dimethoxytrityl. [c] BPO/DMP solution (55%) was diluted with dichloromethane.

Table 4. Phosphoramidite coupling reaction by using ASS **2a**.[a]

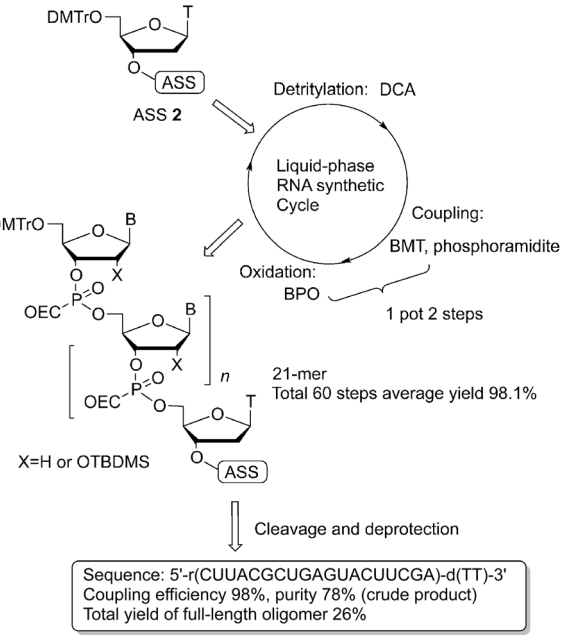


Entry	Phosphoramidite[b]	Solvent	Conversion [%][c]
1	dT	MeCN	3
2	dT	CH_2Cl_2	15
3	dT	MeCN/EtCN/cyclohexane (1:1:10)	5
4	dT	MeCN/CH_2Cl_2 (1:10)	>99
5	rU	MeCN/EtCN/cyclohexane (1:1:10)	5
6	rU	MeCN/CH_2Cl_2 (1:10)	>99

[a] All reactions were performed at RT for 15 min. [b] Phosphoramidite reagent (1.1 equiv) was used in each reactions. [c] Determined by ^{1}H NMR analysis.

(Scheme 1 and Figure 3; 60 steps; recovery yield of fully protected sequence 46%, average 98%; coupling efficiency 98%; full-length oligomer yield 26%). Specifically, this simplified method that precludes redundant synthetic effort, such as capping and the use of molecular sieves, enabled shortening of the recovery step by replacing it with filtration, which resulted in the ability to obtain target products in high yield. In addition, soluble supports, which have high loading capacity (0.92 mol g^{-1}) compared with insoluble resins, such as, for example, macroporous polystyrene, were expected to contribute to creating an effective scale-up manufacturing system.

To monitor each of the ASS-attached oligomers, matrix-assisted laser desorption-ionization time-of-flight mass spectrometry (MALDI-TOF-MS) analysis was carried out without cleaving ASS from the oligomer. Although in this experiment it was not possible to measure RNA-ASS of more than 11-mer in length, 10-mer RNA was detected. It was shown to have high purity, although the peak with the *i*Pac group was partially deprotected (Figure 4). In the assembly of the RNA oligomer, a small amount of acidic reagents, which cannot be removed through the separation process, often leads to side reactions, such as depurination and decomposition of 2′-*O*-TBDMS protecting groups. This result-



Scheme 1. Liquid-phase RNA synthesis by using ASS **2**.

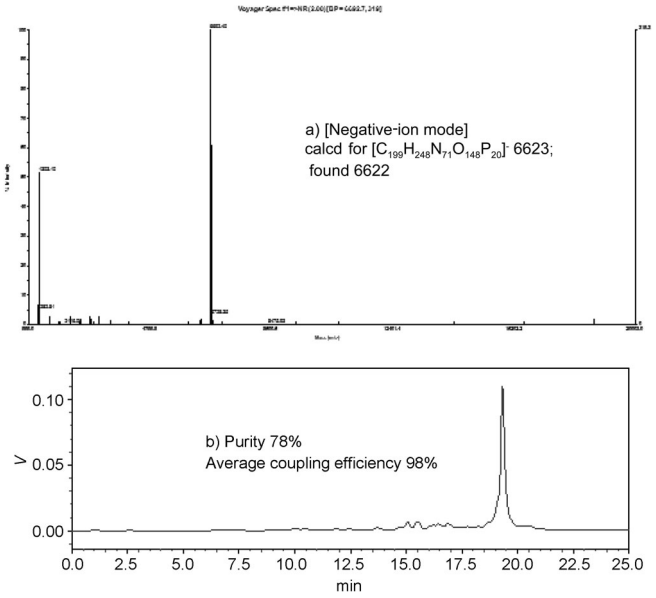


Figure 3. a) MALDI-TOF mass spectrum of full-length RNA oligomer 5′-r(CUUACGCUGAGUACUUCGA)-d(TT)-3′; b) chromatogram of the full-length RNA oligomer. HPLC conditions: column Shodex ODP (4.6 ϕ*150 mm); eluent: gradient A = 0.1 M triethylamine acetic acid, B = acetonitrile (0 to 15%, 25 min); flow rate 1.0 mL min^{-1}; λ = 260 nm; 50 °C.

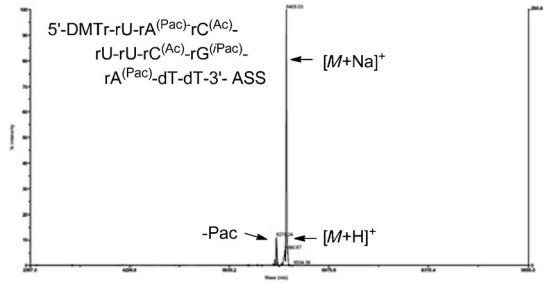


Figure 4. MALDI-TOF mass spectrum of fully protected 10-mer RNA oligomer containing ASS.

ed in the generation of defective sequences and reduced yields. It should be noted that in the liquid-phase synthesis by using ASS developed here, such side-reactions did not occur. It was possible to avoid this side reaction by repeated exposure to multiple reaction and separation steps, which successfully gave the fully protected RNA oligomers containing ASS. On the other hand, during removal of the protecting groups from the fully protected RNA oligomers, some decomposition, such as phosphodiester backbone cleavage, took place, and this diminished the total yield. This resulted from the inherent instability of the RNA oligomer. To overcome this issue in both the solid- and liquid-phase approaches, the post-processing protocol for the large-scale RNA synthesis needs to be improved.

Conclusion

A liquid-phase RNA synthetic system on a gram scale by using an alkyl-chain-soluble support was reported. The dispersion and aggregation of the support can be effectively controlled through a relevant solvent change, allowing for easy separation and high recovery yields. Specifically, the dispersion property was markedly improved by the structural blocking of the hydrogen-bond interactions of the linker moiety and by selection of dispersion media, which enabled enhanced reactivity in the liquid-phase approach. This approach provides a practical and reliable RNA synthetic methodology in the development of scaled-up, cost-effective chemical RNA synthesis. We believe that the applicability of the soluble support and liquid-phase system to the assembly of RNA oligomers holds great promise for the future development of RNA therapeutics.

Experimental Section

General procedure for the liquid-phase reaction by using ASS: ***Detritylation reaction of compound 8***: ASS **2** (1 g, 0.6 mmol) and DCA/dichloromethane solution (5%, 30 mL) was added to dichloromethane (90 mL). After stirring for 1 min, the reaction mixture was diluted with methanol and concentrated in vacuo. The residue was diluted, filtrated, and rinsed with methanol to give ASS **2a** as a white solid (0.95 g, 0.6 mmol, 99%). M.p. 168–169 °C; ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 8.32$ (1H, m), 7.47 (1H, s), 6.57 (2H, s), 6.20 (1H, dd, $J = 8.0$, 6.0 Hz), 5.36 (1H, ddd, $J = 5.6$, 5.2, 2.8 Hz), 4.10 (1H, dd, $J = 4.8$, 2.0 Hz), 3.98–3.91 (6H, m), 3.91–3.85 (2H, m), 3.85–3.55 (8H, m), 2.67 (4H, s), 2.46–2.35 (2H, m), 1.91 (3H, s), 1.81–1.68 (6H, m), 1.50–1.38 (6H, m), 1.35–1.20 (84H, m), 0.86 ppm (9H, t, $J = 7.0$ Hz); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 172.8$, 170.8, 169.9, 163.8, 153.3, 150.5, 139.8, 136.5, 129.7, 111.3, 105.8, 86.0, 85.0, 75.0, 73.6, 69.4, 62.5, 37.1, 31.9, 30.3, 29.7, 29.6, 29.4, 29.3, 29.2, 27.9, 26.1, 22.7, 14.1, 12.6 ppm; IR (KBr): $\tilde{\nu} = 2918$, 2850, 1699, 1637, 1469, 1430, 1123 cm^{-1}; HRMS (ESI$^+$): m/z calcd for $C_{79}H_{138}N_4O_{11}Na$: 1342.0260 [M^+]; found 1342.0872.

Coupling and oxidation reaction of 5′-DMTr-dT-dT-3′-ASS (7): A solution of benzylthio-1*H*-tetrazole/acetonitrile solution (0.25 mol L^{-1}, 7 mL, 1.75 mmol) was added to a solution of ASS **2a** (1.32 g, 1.0 mmol) and amidite monomer (dT-phosphoramidite, 1.12 g, 1.5 mmol) in dry dichloromethane (70 mL) at room temperature. After stirring the resulting reaction mixture for 20 min, 2-butanone peroxide/dichloromethane solution (30 mL) was added. After stirring for 10 min, the resulting solution was diluted with methanol and concentrated in vacuo. The residue was diluted with methanol and filtrated to give the product **7** (1.98 g, 1.0 mmol, 99%) as a white solid. M.p. 132 °C; ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 9.36$ (1H, m), 7.54 and 7.52 (1H, s), 7.40–7.20 (10H, m), 6.84 (4H, d, $J = 8.4$ Hz), 6.59 (2H, s), 6.47–6.35 (1H, m), 6.31–6.20 (1H, m), 5.41–5.24 (1H, m), 5.23–5.13 (1H, m), 4.48–4.09 (6H, m), 4.00–3.88 (6H, m), 3.79 (6H, s), 3.75–3.30 (10H, m), 2.80–2.74 (1H, m), 2.74–2.56 (5H, m), 2.52–2.38 (2H, m), 2.38–2.10 (2H, m), 1.90 (3H, s), 1.84–1.68 (6H, m), 1.53–1.42 (6H, m), 1.41 (3H, s), 1.38–1.08 (84H, m), 0.88 ppm (9H, t, $J = 7.0$ Hz); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 172.7$, 170.7, 169.8, 158.8, 153.3, 150.5, 144.0, 139.8, 135.5, 135.4, 135.0, 130.1, 129.8, 128.6, 128.1, 127.3, 116.4, 116.2, 113.4, 111.8, 111.7, 105.8, 87.3, 85.3, 85.1, 84.5, 84.3, 82.3, 79.9, 79.7, 77.3, 73.8, 73.6, 69.3, 67.8, 63.2, 62.5, 62.4, 55.3, 45.2, 42.0, 39.0, 36.7, 31.9, 29.7, 29.4, 26.1, 22.7, 19.6, 14.1, 12.6, 12.5, 11.7 ppm; ^{31}P NMR (162 MHz, $CDCl_3$): $\delta = -2.854$; IR (KBr): $\tilde{\nu} = 2919$, 2850, 1696, 1650, 1467, 1253, 1117, 1031 cm^{-1}; HRMS (ESI$^+$): m/z calcd for $C_{113}H_{172}N_7O_{20}PNa$: 2001.2292 [$M^+$]; found 2002.3353.

Cleavage and base deprotection: Sterile nuclease-free water was used in all steps. The fully protected sequence (5.85 g, 0.46 mmol) was added to ammonia/water/ethanol solution (28%, 30 mL, 3:1 v/v). The solution was then warmed to 80 °C and stirred for 90 min. The reaction solution was cooled to RT and concentrated in vacuo. NMP, TEA, and TEA-3HF (65 mL, 6:3:4 v/v/v) were added to the residue. After stirring for 90 min at 60 °C, the reaction mixture was cooled to RT. To the solution was added triethylamine/acetic acid buffer (0.1 M, 65 mL, pH 7.4). The full-length RNA oligomer, which contains a 5′-DMTr group, were retained on the Sep-Pak Cartridge (C18–2 g) and washed with water (10 mL). After cleaving the DMTr by TFA (2%, 30 mL) and the reverse-phase resin was washed with water. The full-length RNA oligomer was eluted with MeCN solution (20%). The eluate was dried in centrifugal dryer in vacuo to give the target RNA oligomer (1.72 g, 0.26 mmol).

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[9] See the Supporting Information.

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