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Thio-modification effects on mRNA translation using a PureCap-based capping method

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Controlling translational efficiency, stability, and innate immune recognition remains a critical challenge for the practical application of mRNA therapeutics, and chemical modification of nucleic acids is widely employed as an effective strategy to address these issues. Sugar modifications exemplified by 4′-thio substitution and phosphate-backbone modifications exemplified by phosphorothioate (PS) substitution can alter the behavior of nucleic acids. In this study, to introduce 4′-thio substitution and PS substitution at the +1 and +2 nucleotides of the 5′ end of mRNA, we synthesized a series of cap analogs based on the PureCap method that we previously established, which enables preparation of highly pure capped mRNA for reliable evaluation of cap modification effects. Using the resulting cap analogs, mRNAs were prepared by *in vitro* transcription (IVT) with high-performance liquid chromatography (HPLC) purification, and even when modifications were introduced into the ribose moiety, IVT yields were not substantially reduced. In cultured HeLa cells, PS-modified mRNAs exhibited higher protein expression than the 4′-thio-modified counterparts for Cap 0, Cap 1, and Cap 2 structures. By contrast, for the Cap 2 structure, PureCap-derived mRNAs bearing either 4′-thio or PS modifications showed protein expression comparable to that of an N^1-methylpseudouridine ($m^1\Psi$)-modified control mRNA prepared using the commercially available CleanCap AG, both at 72 h in the cell-based translation assay and at each measurement time point following subcutaneous administration in mice. Taken together, these results suggest that 4′-thio and PS modifications at the 5′-terminal +1 and +2 nucleotides can alter mRNA function. This work further demonstrates the utility of the PureCap platform and highlights its broad applicability for mRNA therapeutic development.

Introduction

Nucleic acid therapeutics hold bright promise as a new treatment modality; however, substantial efforts are still required to improve their efficiency, stability, and immune recognition.[1] In this context, chemical modification has emerged as a particularly important approach.[2] Sulfur (S) and oxygen (O) belong to the same chalcogen group, yet sulfur is characterized by a larger atomic radius and lower electronegativity, properties that can alter the chemical behavior of nucleic acids.[3] Such elemental substitutions also occur in nature: transfer RNA (tRNA) carries multiple sulfur-containing modifications, including 4-thiouridine (s^4U) and 2-thiouridine (s^2U).[4] s^2U is known to suppress miscoding during translation, and s^4U has been reported to enhance tRNA stability.[5–9] Another well-known sulfur modification is the phosphorothioate (PS) modification, in which a non-bridging oxygen atom at the phosphate moiety of the nucleic acid backbone is replaced by sulfur; this modification has been reported to occur naturally in bacterial and archaeal DNA.[10] PS modification is now widely applied to synthetic nucleic acids and nucleic acid therapeutics, where it has been shown to improve nuclease resistance and pharmacokinetic properties.[11] It has become a standard technology in antisense oligonucleotide (ASO) and small interfering RNA (siRNA) therapeutics, and its utility has been demonstrated in numerous FDA-approved drugs, including Nusinersen and Inclisiran.[12–15] On the basis of these successes in oligonucleotide therapeutics, application to mRNA has also advanced.[16] In our previous work, introduction of PS into synthetic nucleic acids accelerated translation initiation in a cell-free translation system and significantly enhanced translation

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efficiency, suggesting the potential applicability of this modification to mRNA therapeutics.[17]

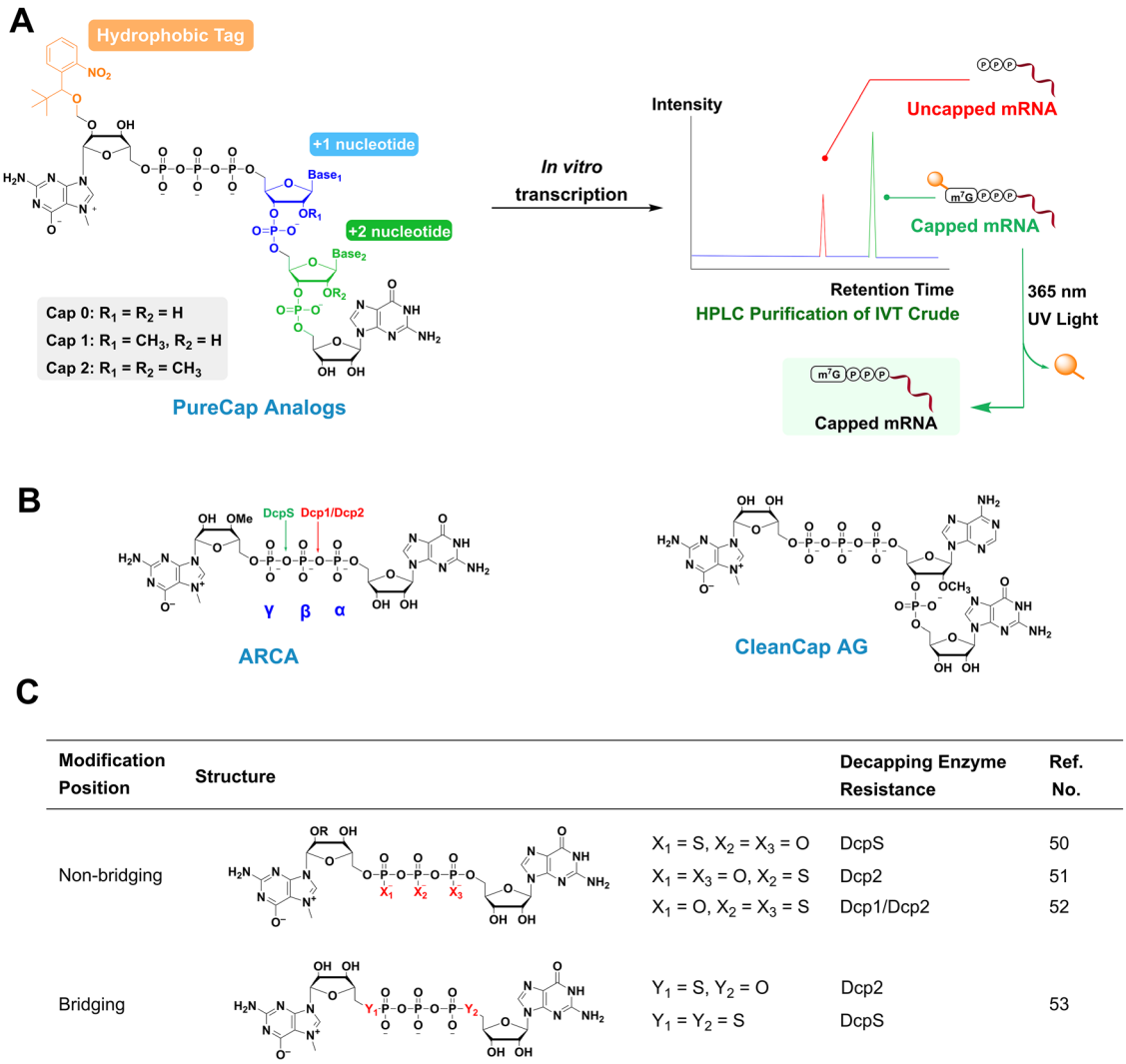
As another example of O-to-S substitution at a different site, 4′-thio-modified nucleic acids, in which the ring oxygen atom of the ribose is replaced by sulfur, are increasingly occupying an important position in next-generation nucleic acid therapeutic development. Replacement of oxygen with sulfur not only perturbs ribose-ring dihedral angles through C–S bond elongation and changes in the electronic structure, but also affects the S4′–C1′–N anomeric effect, thereby improving thermal stability and enhancing nuclease resistance.[18–20] Structural analyses in previous studies have shown that 4′-thio RNA preferentially adopts a C3′-*endo* ribose pucker, as does native RNA, and maintains duplex formation with complementary RNA strands.[18,21] Historically, the synthesis of 4′-thionucleosides has been hampered by multistep and low-yielding processes; however, this situation has been transformed by applications of the Pummerer reaction and improvements to the Reist method.[18,22–24] More recently, scalable and operationally simple approaches have been reported in succession, including one-pot synthesis platforms employing biocatalysts and stereoselective chemical syntheses from acyclic precursors.[25–27] Along with these technological advances, application-oriented studies have diversified. Single-stranded RNAs bearing 4′-thio-locked nucleic acid (4′-thioLNA) modifications exhibit high enzymatic resistance, and in siRNA, synergistic effects between 4′-thio substitution and 2′-modifications such as 2′-*O*-methyl (2′-*O*Me) and 2′-fluoro (2′-F), have been shown to confer enhanced nuclease resistance while maintaining RNA interference activity.[21,28–30] In addition, 4′-thionucleoside triphosphates have been demonstrated to display good substrate compatibility with T7 RNA polymerase (T7 RNAP), enabling the selection of high-performance modified aptamers and improvements in genome-editing efficiency.[31,32] Furthermore, evaluations as antiviral agents, including Remdesivir derivatives against SARS-CoV-2, have been reported, indicating that their utility spans a broad range from small-molecule drug discovery to nucleic acid therapeutics.[33] Collectively, these favorable properties strongly suggest that 4′-thio modification could also contribute to improving the performance of mRNA therapeutics.

mRNA is a therapeutic modality that induces the production of therapeutic proteins *in vivo*, and, following the success of COVID-19 vaccines, its application has been extended toward a broad range of medical uses, including anticancer therapies and protein replacement therapies.[34–36] mRNA comprises a 5′ cap, untranslated regions (UTRs), a coding region, and a poly(A) tail. The 5′ cap contains an N^7-methylguanosine (m^7G) structure and is linked to the mRNA chain through a 5′–5′ triphosphate bridge. Depending on the presence or absence of 2′-*O*-methylation at the first transcribed nucleotide (+1) and the second nucleotide (+2), cap structures are classified as Cap 0, Cap 1, or Cap 2 (Fig. 1A).[37] The 5′ cap is recognized by the eukaryotic translation initiation factor 4E (eIF4E), decapping enzymes (*e.g.*, decapping protein Dcp, decapping scavenger DcpS, and decapping exoribonuclease DXO), and immune-related proteins such as interferon-induced proteins with tetratricopeptide repeats (IFITs) and retinoic acid-inducible gene I (RIG-I), and therefore plays a central role in translation initiation, regulation of mRNA stability, and immune responses.[38–41] Accordingly, the 5′ cap has attracted considerable attention as a key target for controlling and engineering the properties of mRNA.

Capped mRNAs can be synthesized co-transcriptionally by adding small-molecule cap analogs to the reaction mixture (Fig. 1A).[42–45] To prevent misincorporation in the reverse orientation from the m^7G side, anti-reverse cap analogs (ARCAs) bearing a 3′-*O*-Me substitution were reported (Fig. 1B).[46,47] Since then, a variety of chemically modified cap analogs based on ARCA have been developed.[48] Jemielity and co-workers have systematically investigated the effects of O-to-S substitutions within the triphosphate moiety of the cap structure (Fig. 1C). PS substitution at the non-bridging positions (α, β, and γ) within the triphosphate bridge enhances binding affinity to eIF4E.[49,50] Meanwhile, substitution at the β position confers resistance to Dcp2, whereas substitution at the γ position confers resistance to DcpS.[50,51] PS substitution at both the α and β positions was also reported to provide resistance to the Dcp1/2 complex.[52] Furthermore, unlike non-bridging substitutions, replacement of the 5′-O atom at the bridging position with sulfur at the γ position increases resistance to Dcp2, and replacement at both the α and γ positions improves resistance to DcpS.[53] Collectively, these findings indicate that O-to-S substitution at the phosphate moiety can enhance decapping resistance and thereby improve mRNA stability and translational efficiency. In addition to chemical modifications at the phosphate moiety, approaches that alter the cap-analog structure to install a Cap 1 structure during transcription have been developed, with the CleanCap technology being a widely used representative example (Fig. 1B). Such trinucleotide cap analogs are currently broadly employed because they provide high capping efficiency and high translational performance.[54] Moreover, even for trinucleotide cap analogs bearing a PS modification within the triphosphate moiety and possessing a Cap 1 structure, improved translational efficiency has been observed relative to the corresponding Cap 1 trinucleotide cap analog lacking thio-modification, further suggesting the potential utility of thio-substitution.[55]

Despite the advances described above, conventional co-transcriptional capping methods have still been unable to produce quantitatively capped mRNAs with all structural variants. This limitation has made it difficult to accurately assess the effects of chemical modifications. To address this issue, we have recently developed the PureCap method.[56] In this approach, cap analogs bearing a hydrophobic tag on the m^7G moiety enable physicochemical separation of capped mRNA from uncapped mRNA, allowing the isolation of capped mRNA with purity higher than 99% (Fig. 1A).[56] This high capping purity makes the PureCap platform particularly suitable for evaluating the intrinsic effects of chemical modifications on mRNA function without interference from uncapped byproducts.

Previous studies have demonstrated that 4′-thio and PS modifications are effective for controlling the functions of



Modification Position	Structure		Decapping Enzyme Resistance	Ref. No.
Non-bridging		X_1 = S, X_2 = X_3 = O	DcpS	50
		X_1 = X_3 = O, X_2 = S	Dcp2	51
		X_1 = O, X_2 = X_3 = S	Dcp1/Dcp2	52
Bridging		Y_1 = S, Y_2 = O	Dcp2	53
		Y_1 = Y_2 = S	DcpS	

Fig. 1 Development of cap analog compounds. (A) The PureCap method used in this research. Introduction of a hydrophobic tag prolongs the retention time of capped mRNA in chromatography, enabling efficient purification; subsequent photoirradiation yields mRNA with high capping efficiency. (B) Structure of anti-reverse cap analog (ARCA) and CleanCap AG. The α-, β-, and γ-phosphate positions and the decapping enzyme cleavage site are indicated in the structure of the ARCA. (C) Selected cap analogs bearing thiophosphate modifications. The table lists the decapping enzymes that each modification can resist.

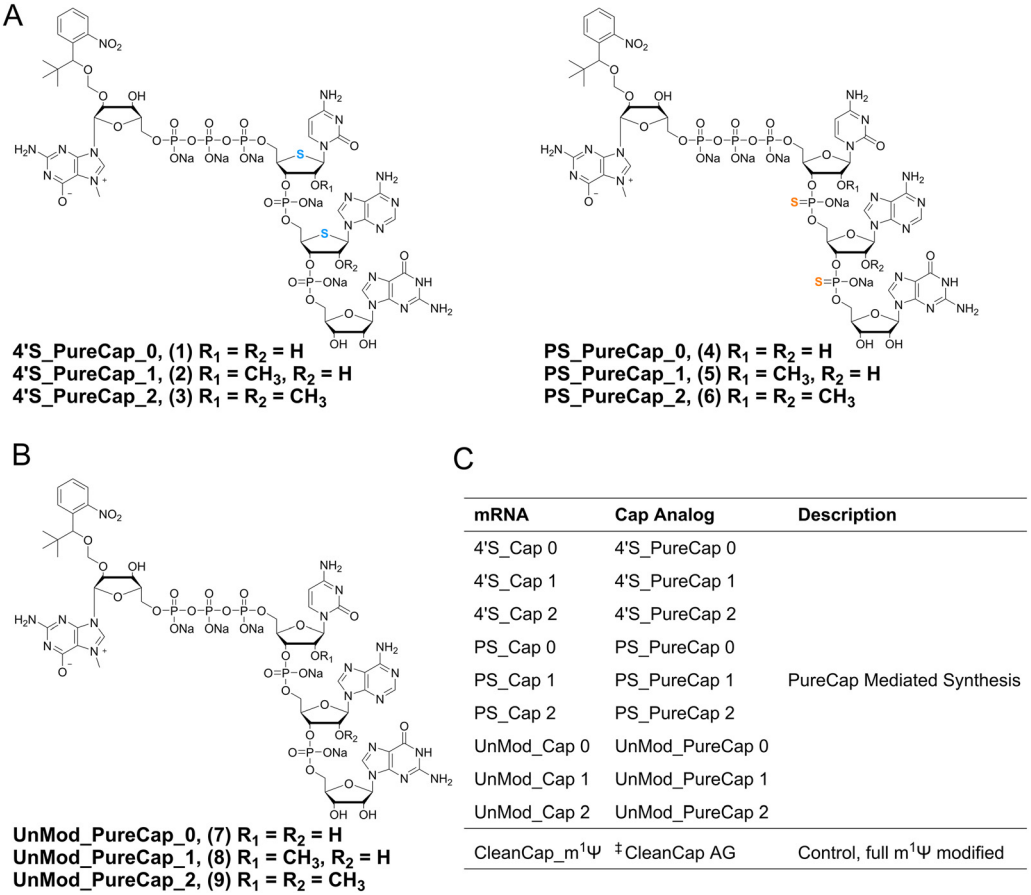
nucleic acid molecules.[31,32,50–53,57] However, studies introducing these modifications into mRNA cap structures remain limited. Moreover, although the impact of modifications at the +1 and +2 positions on translational efficiency has been reported in part, the types of modifications examined have been restricted.[57–60] Because this region is regulated by multiple factors involved in translational control, innate immune responses, and mRNA metabolism, installation of sulfur-based modifications at these positions is expected to provide a new strategy for precise control of mRNA function.

In this study, leveraging the PureCap method, we synthesized cap analogs bearing previously unexplored sulfur substitutions at the sugar ring (4′S modification) and at the backbone (PS modification) at the +1 and +2 positions, and systematically examined their effects on IVT yield, capping efficiency, and translational efficiency in cells and *in vivo*.

Results

Design and synthesis of the cap analogs

In our previous studies, the translational efficiencies of mRNAs bearing Cap 0, Cap 1, and Cap 2 structures have been comparatively evaluated, and the potential advantages of the Cap 2 structure have been highlighted in our results.[56,58,59,61,62] On the basis of this concept, we designed two series of thio-modified cap analogs: a 4′S series (**1**–**3**) and a PS series (**4**–**6**), and synthesized Cap 0, Cap 1, and Cap 2 structures within each series (Fig. 2A). To minimize variables, thio-modifications were introduced at both the +1 and +2 positions in all cap analogs. The corresponding unmodified (UnMod series) Cap 0 (**7**), Cap 1 (**8**), and Cap 2 (**9**) analogs were also synthesized as the controls (Fig. 2B).[56] In the PureCap method, installation of a hydrophobic tag on the cap analog prolongs the retention time of



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mRNA	Cap Analog	Description
4'S_Cap 0	4'S_PureCap 0	
4'S_Cap 1	4'S_PureCap 1	
4'S_Cap 2	4'S_PureCap 2	
PS_Cap 0	PS_PureCap 0	
PS_Cap 1	PS_PureCap 1	PureCap Mediated Synthesis
PS_Cap 2	PS_PureCap 2	
UnMod_Cap 0	UnMod_PureCap 0	
UnMod_Cap 1	UnMod_PureCap 1	
UnMod_Cap 2	UnMod_PureCap 2	
CleanCap_m$^1\Psi$	‡CleanCap AG	Control, full m$^1\Psi$ modified

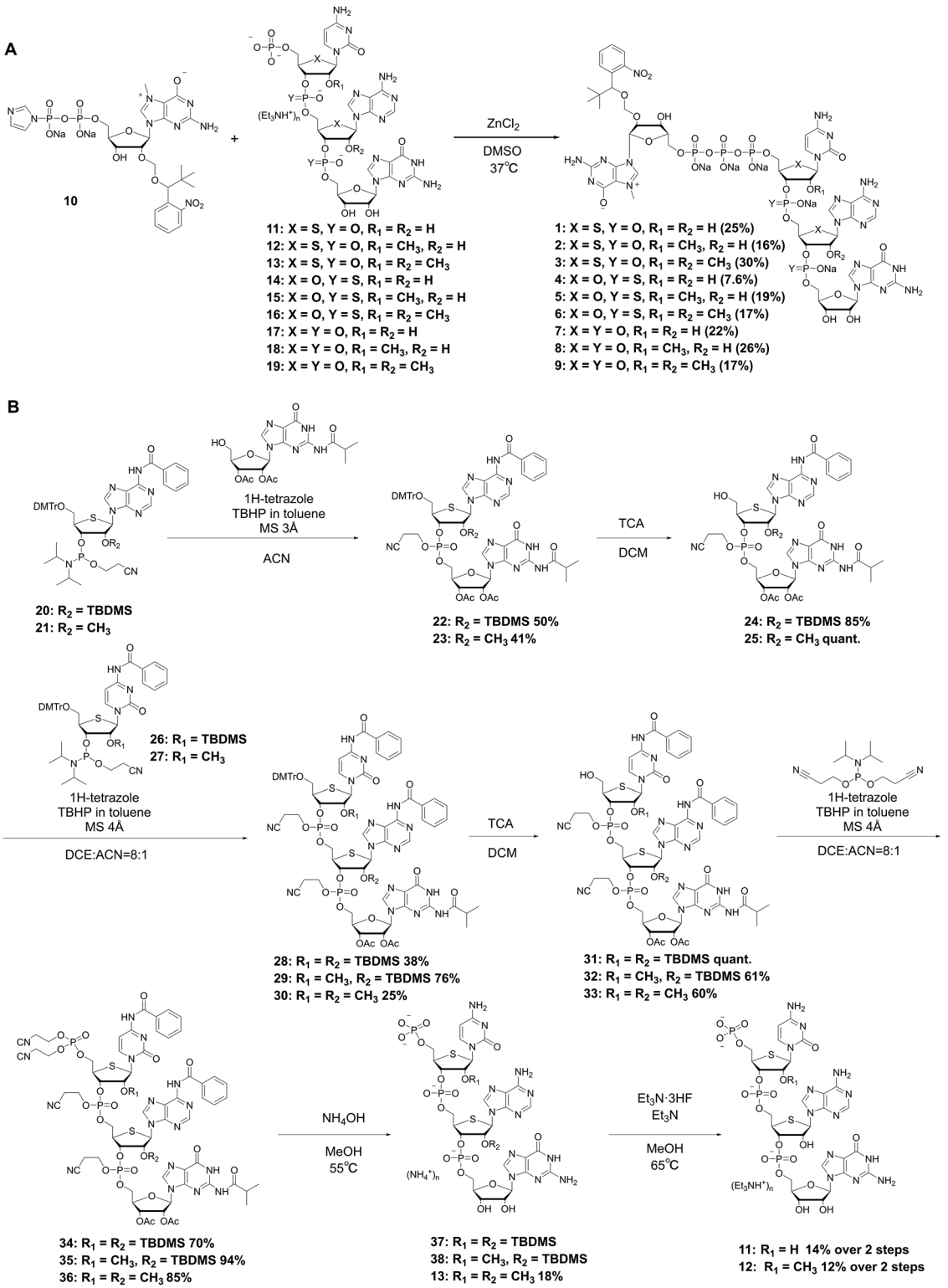
Fig. 2 Cap analog compounds synthesized in this research. (A) Structure of thio-modified-PureCap analogs. (B) Structure of unmodified-PureCap analogs. (C) List of mRNAs and their corresponding cap analogs used in the IVT reactions. ‡A commercially available cap analog as the control.

capped mRNA on reverse-phase HPLC (RP-HPLC), enabling efficient separation from uncapped mRNA.[56] The capped mRNA fraction is then collected, and the hydrophobic tag is subsequently removed by photoirradiation (Fig. 1A).

These PureCap analogs (**1**–**9**) were synthesized by coupling a capping reagent bearing a hydrophobic tag (**10**) with the corresponding trinucleotides (**11**–**19**) (Scheme 1A). The trinucleotides were prepared either by a solution-phase method (Scheme 1B, **11**–**13**) or using a DNA/RNA oligonucleotide synthesizer (**14**–**19**), depending on the desired scale and reagent availability.

In the first step of the solution-phase synthesis of compounds **11**–**13**, the coupling reaction proceeded efficiently in acetonitrile using 2′,3′-protected guanosine as the substrate to achieve 5′-selective coupling, with 1*H*-tetrazole as the activator and molecular sieve (MS) 3 Å present.[63,64] The resulting P(III) intermediate was then oxidized with *tert*-butyl hydroperoxide (TBHP), followed by detritylation with trichloroacetic acid (TCA), to afford the dinucleotides (**24**–**25**). In contrast, in the synthesis of compounds **28**–**30**, the coupling reaction showed little progress under the same conditions. Therefore, the solvent system was changed from acetonitrile to a dichloroethane (DCE)/acetonitrile mixture (v/v = 8 : 1), which markedly improved the reaction efficiency.[64] Subsequent oxidation and detritylation were performed under the same conditions as above. For all synthesized dimers and trimers, the coupling yields after column purification were over 25%. Compounds **34**–**36** were likewise synthesized using 1*H*-tetrazole as the activator in DCE/acetonitrile (v/v = 8 : 1), oxidized with TBHP, and then deprotected by heating in 28% aqueous ammonia/methanol at 55 °C until complete removal of acetyl and base-protecting groups. After the reaction, the solvent was removed by concentration, and desilylation was performed with triethylamine trihydrofluoride (TEA·3HF) in methanol. The trinucleotides (**11**–**13**) were subsequently purified by HPLC to afford the corresponding products in high purity.

The PS and UnMod series trinucleotides (**14**–**19**) were synthesized using an automated oligonucleotide synthesizer, considering time efficiency and synthetic convenience. These trinucleotides were prepared on a 255 μmol scale. For the synthesis of PS-series trinucleotides **14**–**16**, ((dimethylaminomethylidene)amino)-3*H*-1,2,4-dithiazaoline-3-thione (DDTT) was used as the sulfurizing reagent. After synthesis, the dried solid support was suspended in ammonia/methylamine (AMA) solution and incubated at 65 °C for 90 min, followed by filtration and concentration. The resulting crude mixture was dissolved in dimethyl sulfoxide and desilylated with TEA·3HF (DMSO/TEA·3HF, v/v = 1 : 1) at 65 °C for 6 h. Finally, the



Scheme 1 Synthesis of PureCap analogs. (A) Coupling reaction of PureCap analogs. (B) Liquid phase synthesis of compounds **11–13**.

trinucleotides were purified by C18 flash column chromatography and diethylaminoethyl (DEAE) ion-exchange column chromatography.

The cap analogs were further synthesized according to a previously reported method: the purified trinucleotides were reacted with an excess of the capping reagent (**10**; 3–10 equiv.) in the presence of $ZnCl_2$, and the coupling reaction was carried out in DMSO at 37 °C for 2–3 days.[56] The final products were purified by RP-HPLC, converted to the sodium salts, and their purity was verified by liquid chromatography–mass

spectrometry (LC-MS). For all modified cap analogs, the final coupling yields ranged from 7% to 30%, and the purities were higher than 95% (Fig. S1–S9). In the synthesis of **1**–**3**, we examined whether increasing the capping reagent (**10**) up to 10 equivalents would improve coupling efficiency, but no appreciable improvement was observed.

IVT and mRNA sample preparation

The synthesized cap analogs were subsequently used in IVT reactions. We designed a 675 bp double-stranded DNA template containing a T7 RNAP promoter sequence and encoding NanoLuc luciferase (Nluc). To improve capping efficiency, a single complementary adenosine (A) was inserted into the promoter region to complement the cap analog, thereby facilitating efficient initiation with the cap analog and increasing the yield of capped transcripts, following the strategy used in related technologies such as CleanCap (Table S1).[54]

First, to apply these modified PureCap analogs in IVT, we evaluated whether they could serve as substrates for T7 RNAP. Using the PureCap method, capped and uncapped mRNAs are detected as two clearly separated peaks by RP-HPLC (Fig. 1A). By comparing the peak areas with a standard curve, we quantitatively determined mRNA amount (Fig. 3A) and capping efficiency (Fig. 3B). Overall, the 4′S modified cap analogs tended to give slightly lower yields than the corresponding PS and UnMod series analogs with the same cap structure (Cap 0, Cap 1, and Cap 2). In particular, when mRNA was prepared using compound **3**, the yield of capped RNA decreased to approximately 64% and 60% of those obtained using PS-type compound **6** and UnMod-type compound **9**, respectively. Although the yields were somewhat reduced, all values remained within an acceptable range, confirming that these analogs function as substrates for T7 RNAP. This reduction in yield is presumed to arise from ribose conformational changes induced by sulfur substitution at the 4′ position, which may decrease transcription initiation efficiency. In contrast, the capping efficiencies were comparable among all PureCap analogs, with values of 82–85% for the 4′S series, 80–81% for the PS series, and 85–89% for the UnMod series, indicating that these chemical modifications do not significantly affect capping efficiency under the present reaction conditions.

Next, the IVT-prepared mRNAs were isolated and purified by HPLC. After purification, the hydrophobic tag was removed by photoirradiation. A commercially available cap analog, CleanCap AG, was also included as a comparator to assess the performance of PureCap-derived mRNAs relative to an established co-transcriptional capping system. Because $m^1\Psi$ modification is a commonly used approach known to reduce innate immune responses, we prepared an mRNA in which $m^1\Psi$ was incorporated throughout the transcript (CleanCap_$m^1\Psi$) (Fig. 2C).[65–67]

The length and integrity of the purified mRNAs were confirmed by denaturing polyacrylamide gel electrophoresis (dPAGE) (Fig. S10). In addition, the content of double-stranded RNA (dsRNA) was analyzed using a dot blot assay, comparing spot intensities with a poly(I:C) standard (Fig. S11).

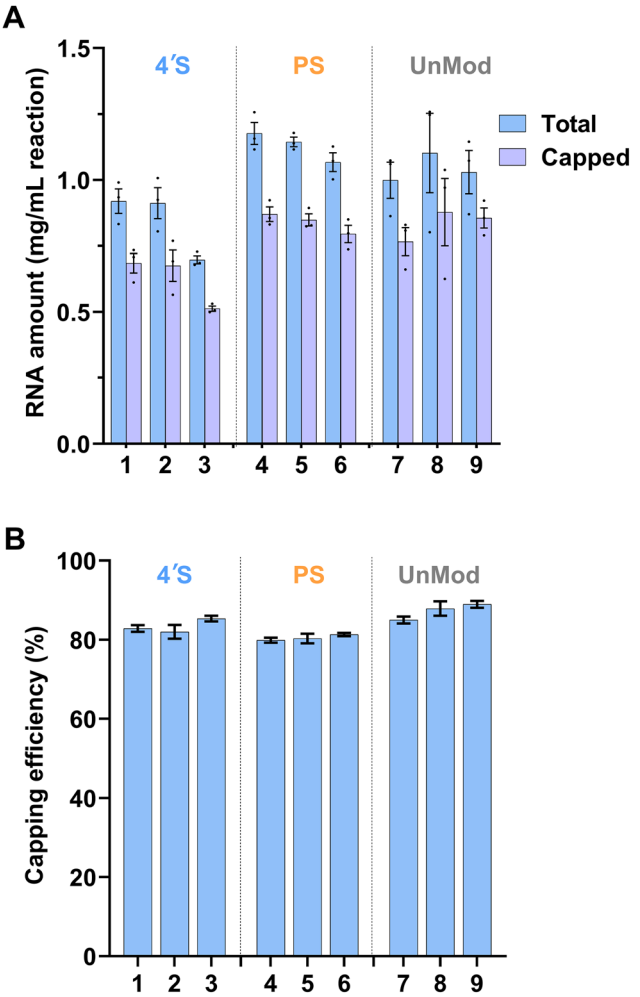


Fig. 3 HPLC-based quantitative analysis of IVT reactions using PureCap analogs. (A) IVT yields quantified by the standard curve analysis. (B) Capping efficiency using various cap analogs. Capping efficiency was calculated as the ratio of peak areas: capped mRNA peak area/total mRNA peak area. The modification types of the caps are indicated above the figure: 4′S (**1**–**3**), PS (**4**–**6**), and UnMod (**7**–**9**); numbers on the *x*-axis represent the cap analogs used (see Fig. 2A and B for their structures). Data are presented as mean ± standard error of the mean (SEM, n = 3).

The dsRNA contents of all PureCap mRNAs showed no significant variation among the samples, suggesting that differences in protein expression are unlikely to arise from dsRNA-dependent suppression of translation through innate immune activation.[68,69] Instead, they are likely attributable to subtle differences in cap structure.

In vitro and *in vivo* translation efficiency

After preparing Nluc mRNAs bearing different cap structures, we first delivered them into cultured HeLa cells using a lipofection reagent, cultured the cells for 6 h, 24 h, 48 h and 72 h, lysed the cells, and quantified mRNA translational activity by measuring the luciferase expression (Fig. 2C and 4).

We first compared the translational activities of the 4′S-, PS-, and UnMod variants within each cap class (Cap 0, Cap 1, and Cap 2). For Cap 0 mRNAs, PS_Cap 0 prepared from

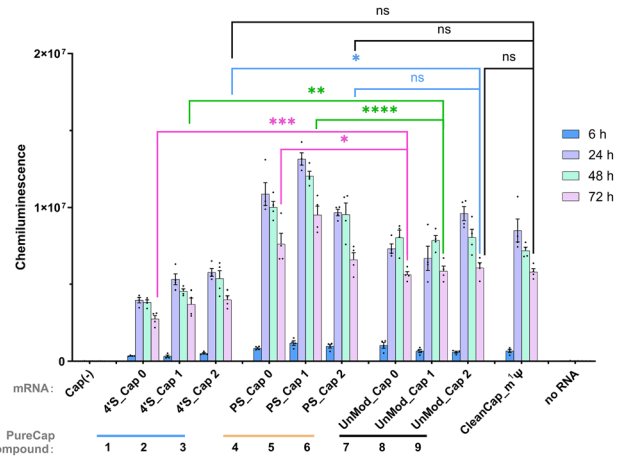


Fig. 4 Translational activity of Nluc mRNA prepared with PureCap analogs and CleanCap AG. mRNAs were transfected into HeLa cells using Lipofectamine MessengerMAX reagent. Cells were cultured for 6 h, 24 h, 48 h or 72 h, lysed, and the Nluc expression was measured. Data are presented as mean ± SEM (n = 4). Statistical significance was determined by one-way ANOVA followed by Tukey's multiple comparisons test to compare mRNA samples within each time point (ns $P > 0.05$, *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$, ****$P \leq 0.0001$).

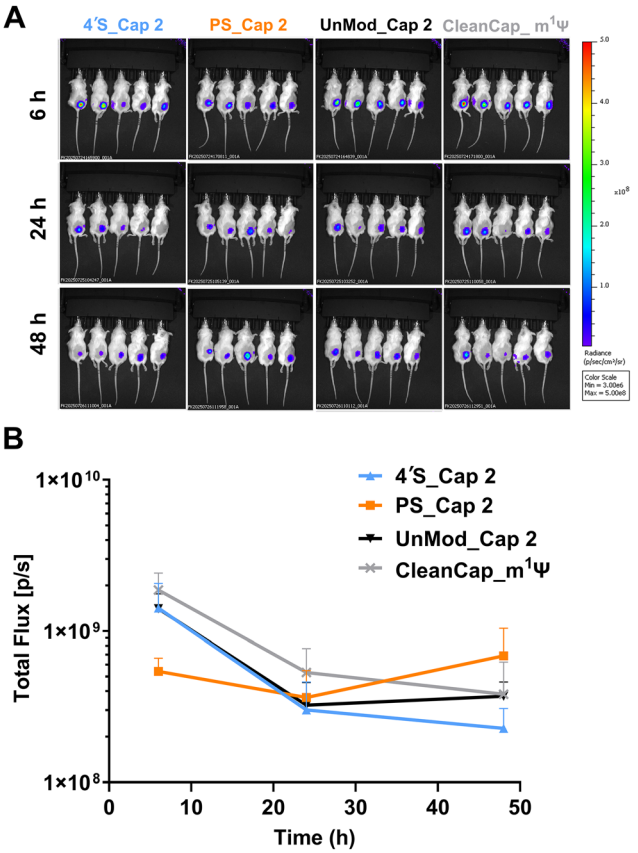


Fig. 5 *In vivo* translation efficiency. (A) Images of mice at 6, 24, and 48 h after subcutaneous administration of 2 μg of Nluc mRNA formulated with LNPs. (B) Total flux (photons per second, p s^{-1}) of mice injected with 2 μg of mRNA encoding Nluc formulated with LNPs for subcutaneous administration (mean + SEM of n = 5). Statistical significance was determined by one-way ANOVA followed by Tukey's multiple comparisons test to compare different samples at each time point. No significant differences were observed between the samples (ns). The y-axis was converted to a logarithmic scale.

compound **4** showed higher protein expression than the corresponding unmodified analog (UnMod_Cap 0 prepared from compound 7), whereas 4′S_Cap 0 prepared from compound **1** showed lower expression at each time point (pink lines). A similar trend was observed for Cap 1 mRNAs at 24 h, 48 h, and 72 h (green lines). In the Cap 2 series, no significant difference in protein expression was observed between PS_Cap 2 derived from compound **6** and the corresponding unmodified analog (UnMod_Cap 2 derived from compound **9**) at each time point, whereas 4′S_Cap 2 derived from compound **3** again showed lower expression than the unmodified analog (blue lines). However, neither 4′S_Cap 2, PS_Cap 2, nor UnMod_Cap 2 showed a significant difference in translational activity compared with CleanCap_m^{1}Ψ at 72 h. We next compared the effects of Cap 0, Cap 1, and Cap 2 structures within each modification series. Within the PS series, PS_Cap 1 showed higher protein expression than PS_Cap 0 and PS_Cap 2 mRNAs at 24 h, 48 h, and 72 h. In contrast, within the UnMod series, UnMod_Cap 2 showed higher protein expression than UnMod_Cap 0 and UnMod_Cap 1 mRNAs only at 24 h, whereas no substantial differences among UnMod_Cap 0, UnMod_Cap 1, and UnMod_Cap 2 were observed at the other time points. In the 4′S series, no apparent differences in protein expression were observed among the three cap structures at any time point. Overall, under the present experimental conditions, the influence of cap structure (Cap 0, Cap 1, and Cap 2) within each modification series appeared to be less substantial than the effects of the chemical modifications themselves.

For the *in vivo* experiments, Cap 2 mRNA encoding Nluc, selected based on our previous finding that Cap 2 structures exhibit higher translational activity *in vivo* than Cap 0 and Cap 1 structures, was encapsulated into lipid nanoparticles (LNPs) using a rapid-mixing method.[56] All mRNAs were formulated under identical LNP preparation conditions and exhibited comparable polydispersity index (PDI, below 0.2), and encapsulation efficiency (EE%, over 90%), ensuring that translation outcomes were evaluated under controlled delivery conditions (Table S2). The resulting mRNA–LNPs were administered subcutaneously into the dorsal region of mice at a dose of 2 μg mRNA per mouse. Nluc expression was measured at 6, 24, and 48 h post-injection (Fig. 5A). Based on *in vivo* luminescence imaging, mRNAs prepared by the PureCap method (4′S_Cap 2, PS_Cap 2 and UnMod_Cap 2) showed protein expression comparable to that of CleanCap_m^{1}Ψ, even though the PureCap-derived mRNAs were composed entirely of natural uridine rather than full-length m^{1}Ψ. Unlike the *in vitro* results, no statistically significant differences were observed among 4′S_Cap 2, PS_Cap 2 and UnMod_Cap 2 at any time point *in vivo*, suggesting that the O-to-S substitutions in the cap structure are accepted *in vivo* (Fig. 5B).

Discussion

In this study, using the PureCap technology, we introduced sulfur-containing modifications into the 5′-terminal cap structure of mRNA. In particular, we designed and synthesized cap analogs (4′S_PureCap and PS_PureCap) bearing distinct modification patterns at the +1 and +2 positions at the 5′ end, and evaluated the translational properties of the mRNAs prepared using these analogs.

Although a wide variety of chemical modifications have been developed for cap structures, there remains substantial room to investigate how site-specific modifications, even within the relatively small structural unit of the cap, affect overall mRNA function. Many previous studies have focused on enhancing eIF4E binding affinity through modifications at the m^7G moiety or on improving resistance to degradation by targeting the triphosphate moiety.[70,71] However, beyond these established modifications, systematic studies examining how chemical modifications downstream of the triphosphate bridge, namely at the +1 and +2 positions, affect the properties of the entire mRNA remain limited. The +1 nucleotide has been shown to influence eIF4E recognition by constraining the orientation of its C-terminal loop region.[72] Indeed, there are reports that modifications at the +1 position, including base modifications such as N^6-methyladenosine (m^6A) and ribose modifications such as 2′-*O*-methoxyethyl (2′-MOE) and LNA, can enhance translational efficiency.[57,58] Moreover, N^6, 2′-*O*-dimethyladenosine (m^6A_m) modification at the +1 position and the Cap 2 structure have been reported to more effectively inhibit complex formation with IFIT proteins than other cap modifications, thereby strengthening recognition as ''self'' mRNA.[59,60] On the other hand, the region around the +1 position is also known as a cleavage site for the decapping enzyme DXO, and chemical modifications of the cap and adjacent nucleotides are therefore considered to be involved in regulation of mRNA stability.[73] Against this background, introducing sulfur-based modifications at the +1 and +2 positions is a possible strategy for modulating mRNA stability, innate immune recognition, and translational activity *via* mechanisms distinct from those of conventional triphosphate modifications.

There have been several reports on representative sulfur modifications, including PS modification and 4′-thioribose modification. For PS modification, examples of introduction at the 5′ end of mRNA have been reported.[50–53,57] In a recent study, differences in the number of introduced PS linkages were shown to affect translational efficiency, suggesting that optimization of sulfur installation proximal to the cap structure may directly contribute to maximizing mRNA function.[57] However, whereas that work employed a multistep workflow combining chemical capping and enzymatic ligation, our approach differs in that the PureCap method enables direct, position-specific introduction of PS modifications at the +1 and +2 positions during the IVT reaction, thereby providing a simpler and more reproducible platform. In contrast, 4′-thio modification exhibits high stability in serum and readily forms stable duplexes, and its applications to siRNA and Cas9 guide sequences have been reported.[21,32] However, to the best of our knowledge, this study is the first to demonstrate that an mRNA bearing a cap structure and carrying site-specific 4′-thioribose modifications can maintain high translational activity in cells and *in vivo*. Our results indicate that chemical modification at the +1 and +2 positions can affect translational efficiency to different extents depending on the structural element modified.

Conclusion

In this study, using the PureCap method, we designed and synthesized Cap 0, Cap 1, and Cap 2-type cap analogs bearing either 4′-thioribose or PS modifications at the +1 and +2 positions, and prepared highly pure mRNAs by IVT followed by HPLC purification. Although the 4′S series showed a modest decrease in IVT yield, capping efficiency was maintained. In translational evaluation in HeLa cells, PS-modified mRNAs, especially Cap 0 and Cap 1 type mRNAs, exhibited higher protein expression than the UnMod and CleanCap_$m^1\Psi$ control, whereas the corresponding 4′S-modified mRNAs showed lower expression levels. However, after LNP encapsulation and subcutaneous administration in mice, 4′S_Cap 2 and PS_Cap 2 showed no significant decrease in protein expression at any measured time point from 6 to 48 h. Collectively, these findings suggest that the +1 and +2 region proximal to the cap represents an additional design space that is independent of the triphosphate moiety, and that installation of sulfur-containing modifications at these positions can modulate mRNA function while preserving translational performance. The PureCap platform is useful in that it enables direct comparison of subtle cap-structure modifications using highly pure mRNAs, and it is expected to facilitate the development of mRNA therapeutic design through future mechanistic validation and optimization, including assessments of resistance to decapping-mediated degradation and impacts on immune responses.

Author contributions

Conceptualization: N. M., H. A.; methodology: Z. M., Y. N., M. I., F. H., N. S.-T., H. A.; investigation: Z. M., Y. N., M. I., Y. K., Y. L., S. K.; data curation: Z. M., Y. K., Y. L.; writing – original draft: Z. M.; writing – review & editing: Y. N., F. H., N. A., Y. K., H. A.; funding acquisition: H. A., Y. K.

Conflicts of interest

The authors declare that they have no conflicts of interest.

Data availability

The data supporting this study are available within the article and its supplementary information (SI). Supplementary information: including experimental procedures, compound characterization data (*e.g.*, NMR, HRMS, and chromatograms), and

additional figures and tables. See DOI: https://doi.org/10.1039/d6cb00047a.

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References

1 X. Sun, S. Setrerrahmane, C. Li, J. Hu and H. Xu, *Signal Transduction Targeted Ther.*, 2024, **9**, 316.
2 A. Liu and X. Wang, *Front. Cell Dev. Biol.*, 2022, **10**, 901510.
3 P. Dande, T. P. Prakash, N. Sioufi, H. Gaus, R. Jarres, A. Berdeja, E. E. Swayze, R. H. Griffey and B. Bhat, *J. Med. Chem.*, 2006, **49**, 1624–1634.
4 Y. Y. Zheng, Y. Wu, T. J. Begley and J. Sheng, *RSC Chem. Biol.*, 2021, **2**, 990–1003.
5 H. Tukenmez, H. Xu, A. Esberg and A. S. Bystrom, *Nucleic Acids Res.*, 2015, **43**, 9489–9499.
6 M. Zhang and Z. Lu, *RNA Biol.*, 2025, **22**, 1–25.
7 N. Shigi, *Front. Genet.*, 2014, **5**, 67.
8 S. Kimura and M. K. Waldor, *Proc. Natl. Acad. Sci. U. S. A.*, 2019, **116**, 1394–1403.
9 J. Urbonavičius, Q. Qian, J. M. B. Durand, T. G. Hagervall and G. R. Björk, *EMBO J.*, 2001, **20**, 4863–4873.
10 L. Wang, S. Jiang, Z. Deng, P. C. Dedon and S. Chen, *FEMS Microbiol. Rev.*, 2019, **43**, 109–122.
11 S. Shukla, C. S. Sumaria and P. I. Pradeepkumar, *ChemMedChem*, 2010, **5**, 328–349.
12 F. Eckstein, *Nucleic Acid Ther.*, 2014, **24**, 374–387.
13 K. Schmidt, T. P. Prakash, A. J. Donner, G. A. Kinberger, H. J. Gaus, A. Low, M. E. Ostergaard, M. Bell, E. E. Swayze and P. P. Seth, *Nucleic Acids Res.*, 2017, **45**, 2294–2306.
14 R. S. Finkel, C. A. Chiriboga, J. Vajsar, J. W. Day, J. Montes, D. C. De Vivo, M. Yamashita, F. Rigo, G. Hung, E. Schneider, D. A. Norris, S. Xia, C. F. Bennett and K. M. Bishop, *Lancet*, 2016, **388**, 3017–3026.
15 K. Fitzgerald, S. White, A. Borodovsky, B. R. Bettencourt, A. Strahs, V. Clausen, P. Wijngaard, J. D. Horton, J. Taubel, A. Brooks, C. Fernando, R. S. Kauffman, D. Kallend, A. Vaishnaw and A. Simon, *N. Engl. J. Med.*, 2017, **376**, 41–51.
16 H. Tohda, N. Chikazumi, T. Ueda, K. Nishikawa and K. Watanabe, *J. Biotechnol.*, 1994, **34**, 61–69.
17 D. Kawaguchi, A. Kodama, N. Abe, K. Takebuchi, F. Hashiya, F. Tomoike, K. Nakamoto, Y. Kimura, Y. Shimizu and H. Abe, *Angew. Chem., Int. Ed.*, 2020, **59**, 17403–17407.
18 P. Haeberli, I. Berger, P. S. Pallan and M. Egli, *Nucleic Acids Res.*, 2005, **33**, 3965–3975.
19 A. Czubatka-Bienkowska, J. Sarnik and T. Poplawski, *Carbohydr. Res.*, 2023, **533**, 108934.
20 L. Bellon, J. L. Barascut, G. Maury, G. Divita, R. Goody and J. L. Imbach, *Nucleic Acids Res.*, 1993, **21**, 1587–1593.
21 M. Takahashi, N. Minakawa and A. Matsuda, *Nucleic Acids Res.*, 2009, **37**, 1353–1362.
22 E. J. Reist, D. E. Gueffroy and L. Goodman, *J. Am. Chem. Soc.*, 1964, **86**, 5658–5663.
23 T. Naka, N. Minakawa, H. Abe, D. Kaga and A. Matsuda, *J. Am. Chem. Soc.*, 2000, **122**, 7233–7243.
24 Y. Yoshimura, K. Kitano, K. Yamada, H. Satoh, M. Watanabe, S. Miura, S. Sakata, T. Sasaki and A. Matsuda, *J. Org. Chem.*, 1997, **62**, 3140–3152.
25 S. Westarp, C. M. M. Benckendorff, J. Motter, V. Rohrs, Y. S. Sanghvi, P. Neubauer, J. Kurreck, A. Kurreck and G. J. Miller, *Angew. Chem., Int. Ed.*, 2024, **63**, e202405040.
26 C. Lucas, E. Fung, M. Nodwell, S. Silverman, B. Singh, L. C. Campeau and R. Britton, *Chem. Sci.*, 2025, **16**, 318–322.
27 C. Eymard, A. Manchoju, A. Almazloum, S. Dostie, M. Prevost, M. Nemer and Y. Guindon, *Molecules*, 2024, **29**, 1647.
28 R. Maeda, N. Saito-Tarashima, H. Wakamatsu, Y. Natori, N. Minakawa and Y. Yoshimura, *Org. Lett.*, 2021, **23**, 4062–4066.
29 J. K. Watts, N. Choubdar, K. Sadalapure, F. Robert, A. S. Wahba, J. Pelletier, B. M. Pinto and M. J. Damha, *Nucleic Acids Res.*, 2007, **35**, 1441–1451.
30 M. Takahashi, C. Nagai, H. Hatakeyama, N. Minakawa, H. Harashima and A. Matsuda, *Nucleic Acids Res.*, 2012, **40**, 5787–5793.
31 Y. Kato, N. Minakawa, N. Ogawa and A. Matsuda, *Nucleic Acids Symp. Ser.*, 2004, **48**, 223–224.
32 N. Saito-Tarashima, M. Ueno, A. Murai, A. Matsuo and N. Minakawa, *Org. Biomol. Chem.*, 2022, **20**, 5245–5248.
33 N. Mihara, Y. Nogi, N. Saito-Tarashima, T. Koma, M. Nomaguchi and N. Minakawa, *Chem. Lett.*, 2025, **54**, upaf080.
34 D. E. Vélez, B. L. Torres and G. Hernández, *Genes*, 2025, **16**, 376.
35 C. Liu, Q. Shi, X. Huang, S. Koo, N. Kong and W. Tao, *Nat. Rev. Cancer*, 2023, **23**, 526–543.
36 T. Vavilis, E. Stamoula, A. Ainatzoglou, A. Sachinidis, M. Lamprinou, I. Dardalas and I. S. Vizirianakis, *Pharmaceutics*, 2023, **15**, 166.
37 Y. Furuichi, M. Morgan, A. J. Shatkin, W. Jelinek, M. Salditt-Georgieff and J. E. Darnell, *Proc. Natl Acad. Sci. USA*, 1975, **72**, 1904–1908.
38 I. Topisirovic, Y. V. Svitkin, N. Sonenberg and A. J. Shatkin, *Wiley Interdiscip. Rev.: RNA*, 2011, **2**, 277–298.
39 E. Grudzien-Nogalska and W. M. Kiledjian, *Wiley Interdiscip. Rev.: RNA*, 2017, **8**, e1379.
40 C. Schuberth-Wagner, J. Ludwig, A. K. Bruder, A. M. Herzner, T. Zillinger, M. Goldeck, T. Schmidt, J. L. Schmid-

Burgk, R. Kerber, S. Wolter, J. P. Stumpel, A. Roth, E. Bartok, C. Drosten, C. Coch, V. Hornung, W. Barchet, B. M. Kummerer, G. Hartmann and M. Schlee, *Immunity*, 2015, **43**, 41–51.

41 Y. M. Abbas, B. T. Laudenbach, S. Martinez-Montero, R. Cencic, M. Habjan, A. Pichlmair, M. J. Damha, J. Pelletier and B. Nagar, *Proc. Natl. Acad. Sci. U. S. A.*, 2017, **114**, E2106–E2115.

42 F. Muttach, N. Muthmann and A. Rentmeister, *Beilstein J. Org. Chem.*, 2017, **13**, 2819–2832.

43 R. Contreras, H. Cheroutre, W. Degrave and W. Fiers, *Nucleic Acids Res.*, 1982, **10**, 6353–6362.

44 M. M. Konarska, R. A. Padgett and P. A. Sharp, *Cell*, 1984, **38**, 731–736.

45 E. Darzynkiewicz, J. Stepinski, I. Ekiel, Y. Jin, D. Haber, T. Sijuwade and S. M. Tahara, *Nucleic Acids Res.*, 1988, **16**, 8953–8962.

46 J. Jemielity, T. Fowler, J. Zuberek, J. Stepinski, M. Lewdorowicz, A. Niedzwiecka, R. Stolarski, E. Darzynkiewicz and R. E. Rhoads, *RNA*, 2003, **9**, 1108–1122.

47 E. Grudzien, J. Stepinski, M. Jankowska-Anyszka, R. Stolarski, E. Darzynkiewicz and R. E. Rhoads, *RNA*, 2004, **10**, 1479–1487.

48 M. Shanmugasundaram, A. Senthilvelan and A. R. Kore, *Chem. Rec.*, 2022, **22**, e202200005.

49 M. Warminski, J. Kowalska, E. Nowak, D. Kubacka, R. Tibble, R. Kasprzyk, P. J. Sikorski, J. D. Gross, M. Nowotny and J. Jemielity, *ACS Chem. Biol.*, 2021, **16**, 334–343.

50 J. Kowalska, M. Lewdorowicz, J. Zuberek, E. Grudzien-Nogalska, E. Bojarska, J. Stepinski, R. E. Rhoads, E. Darzynkiewicz, R. E. Davis and J. Jemielity, *RNA*, 2008, **14**, 1119–1131.

51 E. Grudzien-Nogalska, J. Jemielity, J. Kowalska, E. Darzynkiewicz and R. E. Rhoads, *RNA*, 2007, **13**, 1745–1755.

52 M. Strenkowska, R. Grzela, M. Majewski, K. Wnek, J. Kowalska, M. Lukaszewicz, J. Zuberek, E. Darzynkiewicz, A. N. Kuhn, U. Sahin and J. Jemielity, *Nucleic Acids Res.*, 2016, **44**, 9578–9590.

53 B. A. Wojtczak, P. J. Sikorski, K. Fac-Dabrowska, A. Nowicka, M. Warminski, D. Kubacka, E. Nowak, M. Nowotny, J. Kowalska and J. Jemielity, *J. Am. Chem. Soc.*, 2018, **140**, 5987–5999.

54 J. M. Henderson, A. Ujita, E. Hill, S. Yousif-Rosales, C. Smith, N. Ko, T. McReynolds, C. R. Cabral, J. R. Escamilla-Powers and M. E. Houston, *Curr. Protoc.*, 2021, **1**, e39.

55 M. Warminski, A. Depaix, K. Ziemkiewicz, T. Spiewla, J. Zuberek, K. Drazkowska, H. Kedzierska, A. Popielec, M. R. Baranowski, M. Sklucka, M. Bednarczyk, M. Smietanski, K. Wolosewicz, B. Majewski, R. A. Serwa, D. Nowis, J. Golab, J. Kowalska and J. Jemielity, *Nucleic Acids Res.*, 2024, **52**, 10788–10809.

56 M. Inagaki, N. Abe, Z. Li, Y. Nakashima, S. Acharyya, K. Ogawa, D. Kawaguchi, H. Hiraoka, A. Banno, Z. Meng, M. Tada, T. Ishida, P. Lyu, K. Kokubo, H. Murase, F. Hashiya, Y. Kimura, S. Uchida and H. Abe, *Nat. Commun.*, 2023, **14**, 2657.

57 H. Chen, D. Liu, A. Aditham, J. Guo, J. Huang, F. Kostas, K. Maher, M. J. Friedrich, R. J. Xavier, F. Zhang and X. Wang, *Nat. Biotechnol.*, 2025, **43**, 1128–1143.

58 P. J. Sikorski, M. Warminski, D. Kubacka, T. Ratajczak, D. Nowis, J. Kowalska and J. Jemielity, *Nucleic Acids Res.*, 2020, **48**, 1607–1626.

59 K. Drazkowska, R. Tomecki, M. Warminski, N. Baran, D. Cysewski, A. Depaix, R. Kasprzyk, J. Kowalska, J. Jemielity and P. J. Sikorski, *Nucleic Acids Res.*, 2022, **50**, 9051–9071.

60 J. Geng, M. Chrabaszczewska, K. Kurpiejewski, A. Stankiewicz-Drogon, M. Jankowska-Anyszka, E. Darzynkiewicz and R. Grzela, *RNA*, 2024, **30**, 1292–1305.

61 V. Despic and S. R. Jaffrey, *Nature*, 2023, **614**, 358–366.

62 N. Abe, A. Imaeda, M. Inagaki, Z. Li, D. Kawaguchi, K. Onda, Y. Nakashima, S. Uchida, F. Hashiya, Y. Kimura and H. Abe, *ACS Chem. Biol.*, 2022, **17**, 1308–1314.

63 A. Senthilvelan, T. Vonderfecht, M. Shanmugasundaram, I. Pal, J. Potter and A. R. Kore, *Org. Lett.*, 2021, **23**, 4133–4136.

64 N. Saito-Tarashima, M. Kinoshita, Y. Igata, Y. Kashiwabara and N. Minakawa, *RSC Med. Chem.*, 2021, **12**, 1519–1524.

65 K. D. Nance and J. L. Meier, *ACS Cent. Sci.*, 2021, **7**, 748–756.

66 N. Pardi, S. Tuyishime, H. Muramatsu, K. Kariko, B. L. Mui, Y. K. Tam, T. D. Madden, M. J. Hope and D. Weissman, *J. Controlled Release*, 2015, **217**, 345–351.

67 N. Pardi, M. J. Hogan, R. S. Pelc, H. Muramatsu, H. Andersen, C. R. DeMaso, K. A. Dowd, L. L. Sutherland, R. M. Scearce, R. Parks, W. Wagner, A. Granados, J. Greenhouse, M. Walker, E. Willis, J. S. Yu, C. E. McGee, G. D. Sempowski, B. L. Mui, Y. K. Tam, Y. J. Huang, D. Vanlandingham, V. M. Holmes, H. Balachandran, S. Sahu, M. Lifton, S. Higgs, S. E. Hensley, T. D. Madden, M. J. Hope, K. Kariko, S. Santra, B. S. Graham, M. G. Lewis, T. C. Pierson, B. F. Haynes and D. Weissman, *Nature*, 2017, **543**, 248–251.

68 Y. Y. Siew and W. Zhang, *J. Chromatogr. A*, 2025, **1740**, 465576.

69 K. Kariko, H. Muramatsu, J. Ludwig and D. Weissman, *Nucleic Acids Res.*, 2011, **39**, e142.

70 K. Kurpiejewski, K. Piecyk, M. Lukaszewicz, K. Kamel, K. Chmurski, S. Kmiecik and M. Jankowska-Anyszka, *Pharmaceuticals*, 2024, **17**, 632.

71 A. M. Rydzik, M. Warminski, P. J. Sikorski, M. R. Baranowski, S. Walczak, J. Kowalska, J. Zuberek, M. Lukaszewicz, E. Nowak, T. D. W. Claridge, E. Darzynkiewicz, M. Nowotny and J. Jemielity, *Nucleic Acids Res.*, 2017, **45**, 8661–8675.

72 K. Tomoo, X. Shen, K. Okabe, Y. Nozoe, S. Fukuhara, S. Morino, T. Ishida, T. Taniguchi, H. Hasegawa, A. Terashima, M. Sasaki, Y. Katsuya, K. Kitamura, H. Miyoshi, M. Ishikawa and K. Miura, *Biochem. J.*, 2002, **362**(Pt 3), 539–544.

73 X. Jiao, J. H. Chang, T. Kilic, L. Tong and M. Kiledjian, *Mol. Cell*, 2013, **50**, 104–115.