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ARTICLE

Sydnone-modified nucleosides as versatile tools for bioorthogonal post-synthetic functionalization of antisense oligonucleotides

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The development of efficient bioconjugation methods is essential for enhancing the therapeutic potential of oligonucleotides, especially antisense oligonucleotides (ASOs). Two main strategies are used for modification during oligonucleotide solid-phase synthesis: the early incorporation of functionalized monomers, or the post-synthetic modification of precursors bearing small reactive groups—the latter offering greater versatility and yield. Extensive efforts have been dedicated to incorporating bioorthogonal groups into phosphoramidite building blocks to enable the controlled chemical synthesis of reactive oligonucleotides suitable for post-synthetic modifications. Among these, alkynes and cyclooctynes are the most widely used, enabling Cu(I)-catalyzed azide–alkyne cycloaddition (CuAAC) and strain-promoted azide–alkyne cycloaddition (SPAAC), respectively. We recently demonstrated the compatibility of the sydnone group with automated solid-phase chemistry. This chemical motif shows significant promise in oligonucleotide chemistry, as it allows for strain-promoted sydnone–alkyne cycloaddition (SPSAC), a reaction that has recently emerged as an efficient alternative to SPAAC for cellular studies. Herein, we present the synthesis of three sydnone-functionalized phosphoramidite monomers, their incorporation into ASOs, and an evaluation of their chemical and biological properties. These developments aim to expand the bioconjugation toolbox for ASO tracking, targeting, and imaging, thereby improving their therapeutic application and the understanding of intracellular mechanisms.

Introduction

Given the growing interest in therapeutic oligonucleotides, the development of efficient bioconjugation methods is a crucial area of research. These methods enable the attachment of various compounds - such as imaging agents, targeting molecules or transfection agents - onto oligonucleotides, and have diverse applications in improving the delivery and potency of these therapeutic oligonucleotides.[1,2] In solid-phase oligonucleotide synthesis (SPOS), two main strategies can be used to introduce functional species into oligonucleotides: (i) incorporating a nucleoside phosphoramidite bearing the desired functionality early in the process, prior to oligomerization; or (ii) carrying out a post-synthetic modification of a nucleoside that contains a suitable reactive handle. In the first approach, the modified monomer must be sufficiently robust to withstand the harsh chemical conditions of SPOS. The second approach provides a more versatile and efficient way to synthesize various conjugated oligonucleotides from a single common precursor. Indeed, minor modifications to the nucleoside are generally not expected to have a significant impact on the overall yield of the synthesis. This method enables the subsequent functionalization of the oligonucleotide with the desired reactive moiety, typically resulting in improved overall yields.

Among the chemical strategies available for post-synthetic conjugation, bioorthogonal chemistry stands out as particularly robust and versatile. It enables highly efficient and selective chemical modifications of oligonucleotides *in vitro*, within cells, or even in living organisms. Consequently, a wide range of DNA or RNA phosphoramidite building blocks containing classical bioorthogonal groups have been developed, along with bioorthogonal triphosphate nucleosides that can be recognized and processed by polymerases.[3,4]

Among the bioorthogonal chemical functions compatible with the harsh conditions of SPOS, terminal alkynes and cyclooctynes are the most widely used. Terminal alkynes enable CuAAC with azide tags, a commonly used reaction due to its fast kinetics and high efficiency. Several alkyne-modified nucleosides have been synthesized as phosphoramidites and subsequently applied in chemical oligonucleotide synthesis. These modifications have been introduced either on the nucleobase (at the C-5 position of pyrimidines or the C-7 position of 7-deazapurines) or on the 2'-OH position of the ribose.[5-9] Post-synthetic backbone functionalization via CuAAC was also achieved using an alkynyl

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phosphinoamidite, which is compatible with standard SPOS.[10] Phosphoramidite building blocks with cyclooctynes (e.g., DIFO[11], DIBO,[12-14] BCN[12] and COMBO[15]) grafted to either the nucleobase, ribose, or phosphate backbone have also been developed, allowing functionalization with azide compounds via SPAAC. In contrast, the preparation of azide-functionalized oligonucleotides for post-functionalization via CuAAC or SPAAC reactions is more challenging. Although azide functionalities can be incorporated into oligonucleotides, their use remains challenging under standard SPOS conditions and often requires adapted strategies, due to their limited stability and side reactivity with phosphorus (III) species. Alternatively, building blocks with P(V) and azido-modified nucleosides can be incorporated using the phosphotriester method.[16,17] More recently, Micura introduced a robust method for producing azido-modified oligonucleotides by converting amine groups to azides through diazotransfer reactions on oligonucleotides.[18,19] Several nucleosides containing dienes or dienophiles have been reported, allowing internal labelling through inverse electron demand Diels-Alder (iEDDA) reactions[20]. While 1,2,4,5-tetrazine derivatives are well established in bioorthogonal chemistry, their use in oligonucleotide chemical synthesis remains limited.[4] Dienophiles such as trans-cyclooctene (TCO),[21] norbornene,[22,23] and more recently, methylcyclopropene[24,25] have been successfully incorporated into nucleosides as phosphoramidite building blocks and used for SPOS. Notably, for particularly sensitive dienophiles such as TCO and methylcyclopropene, alternative oxidation conditions instead of the standard aqueous iodine treatment may be required to preserve their structural integrity during oligonucleotide synthesis.[26]

Other bioorthogonal groups have already been introduced through polymerase approaches using nucleoside triphosphate building blocks.[4] Depending on the application, the chemical synthesis of oligonucleotides offers, however, several advantages over enzymatic methods: it is less constrained by scale and provides precise control over the sequence, including the position and number of incorporated modifications. In the field of therapeutic oligonucleotides, especially antisense oligonucleotides (ASOs), robust and reliable synthetic methods are required, as they often feature multiple chemical modifications to improve nuclease resistance, target affinity, and penetration properties.[27] ASOs hybridize with high specificity to their target mRNA, thereby modulating gene expression.[28] Among them, gapmer ASOs are built with a central gap of 8 to 10 deoxyribonucleotides that activate RNase H, flanked on both sides by 3 to 5 nucleotides, which are often chemically modified at the 2' sugar position to enhance mRNA affinity.[29,30] They operate via a degradation-dependent mechanism, in which the RNA/DNA heteroduplex recruits RNase H, leading to the degradation of the target mRNA. In recent years, several limitations associated with the therapeutic use of ASOs have been overcome through chemical innovation, particularly in improving mRNA affinity, pharmacokinetics, and pharmacodynamics.[31] One of the major challenges in antisense therapy is enhancing the targeting and cellular uptake of ASOs.[32,33] Equally important is the ability to monitor their internalization and intracellular localization, which is essential for elucidating uptake mechanisms and modes of action.

There are still few described examples of ASO conjugation using these bioorthogonal reactions. Among them, a leukemia-targeting compound composed of a monoclonal antibody conjugated to an ASO through a SPAAC reaction was reported.[34] More recently, *in vivo* click chemistry was used for non-invasive imaging of a TCO-conjugated ASO in the brain using an emerging strategy known as "pretargeted" imaging.[35] A ^{18}F-based tracer with a tetrazine moiety is injected in a second step to undergo an *in vivo* click reaction through an iEDDA reaction. In another recent study, ASOs functionalized at the 5' end with 4-halogeno-sydnones were investigated both to extend bioconjugation strategies at the terminal position and to serve as a useful tag in nanoscale secondary ion mass spectrometry imaging (NanoSIMS).[36]

With the aim of providing new tools for the study of ASOs, we are interested in the development of new bioconjugation methods that employ fast and efficient bioorthogonal reactions for conjugating various biological or imaging entities. In particular, strategies that enable internal conjugation may offer greater stability within the cellular environment by providing enhanced resistance to exonucleases. In a recent study, we turned our attention to SPSAC[37,38,39]. Regarding kinetics in the context of oligonucleotide bioconjugation, we previously reported comparable SPSAC and iEDDA (using cyclopropene as the dienophile) rate constants of $k = 12.6\ M^{-1}{\cdot}s^{-1}$ and $k \approx 8.4\ M^{-1}{\cdot}s^{-1}$, respectively. Beyond intrinsic kinetics, practical considerations related to stability and compatibility with oligonucleotide synthesis are also important. In this regard, SPSAC offers several complementary advantages, notably the stability and accessibility of its reactive partners, the ability to fine-tune reactivity through sydnone halogenation, and its proven efficiency in complex biological environments. Conversely, tetrazine-based iEDDA systems can suffer from limited compatibility with SPOS, and additional challenges may arise with certain dienophiles during oxidation steps. Taken together, these elements highlight SPSAC as an attractive alternative to both SPAAC and iEDDA for cellular oligonucleotide labeling applications.

In a previous study, we successfully synthesized a new phosphoramidite building block featuring an aryl-sydnone moiety attached *via* an aminoethoxy linker to the 2'-*O*-position of a ribothymidine. Interestingly, we demonstrated its compatibility with SPOS conditions (Figure 1A). Moreover, we showed that the kinetics of post-synthetic functionalization with a DBCO fluorescent probe is suitable for further studies in cells.[24] Interestingly, a proof-of-concept study by Wagenknecht et al. with oligonucleotides featuring a post-synthetically introduced sydnone demonstrated their feasibility for use in cells.[40]

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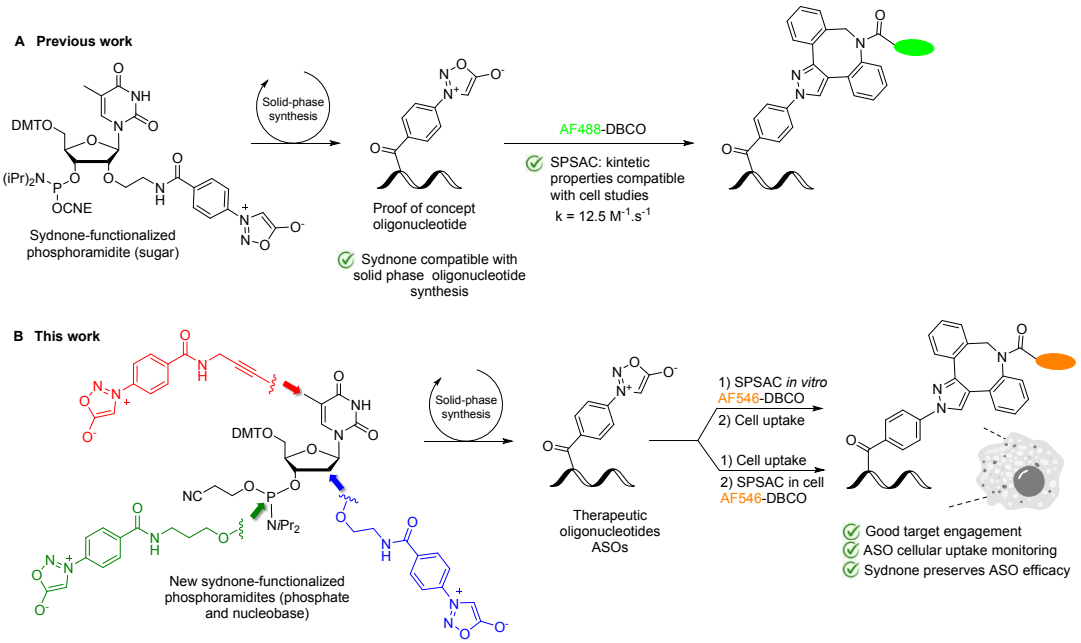


Figure 1. Incorporation of sydnones into oligonucleotides by SPOS for strain-promoted sydnone–alkyne cycloaddition (SPSAC) based bioconjugation.

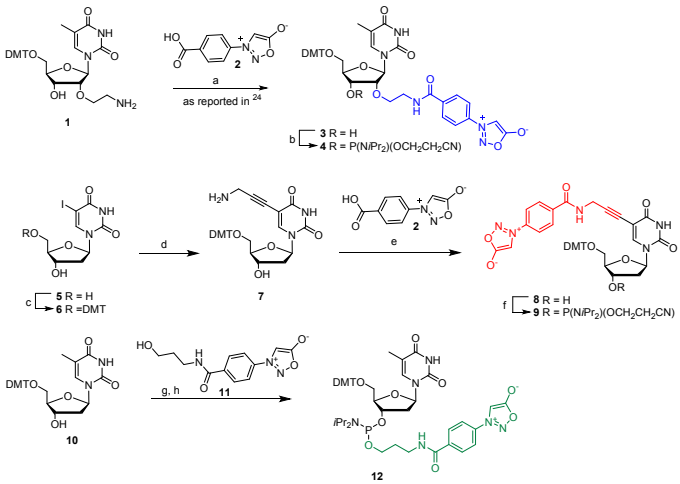
To explore the versatility of the sydnone moiety and its potential for bioorthogonal functionalization of ASOs, we report (i) the synthesis of two new phosphoramidite building blocks bearing a sydnone group on the phosphorus, or nucleobase core completing our previous ribose functionalized phosphoramidite ; (ii) their use as monomers for internal or external incorporation in solid-phase oligonucleotide synthesis; (iii) the synthesis of several ASOs containing the sydnone moiety that enables bioconjugation using SPSAC at the phosphate group, the 2'-OH position of the ribose sugar, or the C-5 position of thymine; and (iv) an investigation of their properties and cellular applications (Figure 1B).

RESULTS AND DISCUSSION

Synthesis of sydnone-based monomers for solid-phase oligonucleotide synthesis

The phenylsydnone moiety was introduced at three distinct positions: on the ribose (at the 2'-oxygen position via an amidoethoxy linker), on the nucleobase (at the C-5-position via a propargyl linker), or on the phosphorus atom of thymidine (via an amidoethoxy linker) (Scheme 1).

We have previously reported the synthesis of the 2'-functionalized phosphoramidite monomer **4**.[24] Briefly, it was obtained from the amine intermediate **1** according to a procedure described by Brown.[41,42] The functionalized phenylsydnone amide **3** was prepared in 72% yield by co upling phenylsydnone acid **2** (prepared from 2-aminobenzoic acid according to reported procedures[44,45]) with compound **1**. Subsequent phosphitylation of **3** afforded the 2'-O-Syd-T phosphoramidite monomer **4** in 74% yield.



Scheme 1. Synthesis of sydnone-based phosphoramidite building blocks **4**, **9** and **12**. Reagents and conditions: a) **2**, 2-(1H-Benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU), DIPEA, DMF, rt, 1 h, 66%; b) 2-cyanoethyl *N,N,N',N'*-tetraisopropylphosphorodiamidite, diisopropylammonium tetrazolide, CH_2Cl_2, rt, 3 h, 74%; c) DMTCl, pyridine, rt, 5 h 91%; d) propargylamine, CuI, $Pd(PPh_3)_4$, Et_3N, DMF, rt, 2.5 h, 67%; e) **2**, TBTU, DIPEA, DMF, rt, 18 h, 75%; f) 2-cyanoethyl *N,N,N',N'*-tetraisopropylphosphorodiamidite, diisopropylammonium tetrazolide, CH_2Cl_2, rt, 3 h, 51%; g) bis(*N,N*-diisopropylamino)chlorophosphine, Et_3N, dioxane, rt, 20 min, filtration, evaporation; h) **11**, ethylthiotetrazole, CH_2Cl_2:CH_3CN, rt, 30 min, 49%.

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The C5-functionalized phosphoramidite monomer **9** was synthesized in a good overall yield. Tritylation of commercially available 5-iodo-deoxyuridine **5** followed by Sonogashira cross-coupling of **6** with propargylamine furnished compound **7**[12] in 67% yield. Coupling of **7** with phenylsydnone acid **2** provided compound **8** in 73% yield, which upon phosphitylation gave the 5-Syd-dT phosphoramidite monomer **9**. The phosphorus functionalization was performed according to the van Boom procedure.[45] Treatment of **10** with bis(*N,N*-diisopropylamino)chlorophosphine afforded the corresponding phosphorodiamidite, which was subsequently engaged in a reaction with phenylsydnone **11** conjugated to an aminopropanol linker in the presence of ethylthiotetrazole to afford the P-Syd-dT phosphoramidite monomer **12** in 49% yield.

Incorporation of sydnone-based nucleosides into antisense oligonucleotides

We prepared 20-mer gapmer ASOs targeting the long non-coding RNA Metastasis-Associated Lung Adenocarcinoma Transcript 1 (MALAT1). The sydnone-based nucleoside **4** was incorporated into the flanking regions (int-2'-Syd-ASO and ext-2'-Syd-ASO), while the sydnone-based nucleosides **9** and **12** were incorporated into the gap region (5-Syd-ASO and P-Syd-ASO, respectively) of ASOs containing 2'MOE-nucleosides in the flanking regions, deoxynucleosides in the gap, and phosphorothioate linkages. The oligonucleotides were generated by means of solid-phase oligonucleotide synthesis using the phosphoramidite method and standard coupling conditions (Table 1).

The results showed that phosphoramidites **4**, **9** and **12** were successfully incorporated into the ASO sequences. In all cases, there was no significant impact on synthesis yields. These results demonstrate that the sydnone motif is a versatile and robust bioorthogonal handle compatible with standard SPOS, including phosphorothioate-based ASOs. It can be incorporated at various positions within the oligonucleotide sequence as well as at different locations on the monomer, enabling site-specific conjugation strategies that may target either the major or minor groove of a duplex.

Functionalization of sydnone-modified ASOs by SPSAC

To study post-synthetic functionalization, we used an AF546 fluorophore containing a DBCO reactive group, which was successfully conjugated in good yields to the previously obtained sydnone-modified ASOs via a SPSAC reaction in phosphate buffer at 37 °C (Table 2 and Supporting Information). While slight variations in yields were observed (55–70%), depending on the sydnone position onto the nucleoside (ribose, nucleobase, or phosphodiester linker) and along the sequence (internal (wing or gap) or terminal), no clear trend or strong positional effect on the SPSAC reaction efficiency was identified. The reaction rates were consistent with those previously reported for 12-mer DNA or RNA oligonucleotides.[24]

Oligonucleotides	Sequences	Yield[a] (nmol)
Ref-ASO	**T*G*C*C*T***T*T*T*A*G*G*A*T*T*C*T***A*G*A*C*A**	54.5
Int-2'-Syd-ASO	**T*G*C*C*T**$^{(2'\text{-Syd})}$*T*T*A*G*G*A*T*T*C*T***A*G*A*C*A**	48.7[b]
Ext-2'-Syd-ASO	$^{(2'\text{-Syd})}$**T*G*C*C*T***T*T*T*A*G*G*A*T*T*C*T***A*G*A*C*A**	215[b]
5-Syd-ASO	**T*G*C*C*T***T*T*T*A*G*G*A*T**$^{(5\text{-Syd})}$*T*C*T***A*G*A*C*A**	88.1[b]
P-Syd-ASO	**T*G*C*C*T***T*T*T*A*G*G*A*T**$^{(P\text{-Syd})}$*T*C*T***A*G*A*C*A**	107[c]

Table 1. ASO sequences and synthesis yields. [a] On a 1 µmol scale after HPLC purification, Syd-phosphoramidite coupling conditions: [b] Syd-phosphoramidite at 0.1 M in acetonitrile, benzylthiotetrazole (BTT) 0.25 M in acetonitrile, 15-20 min; [c] Syd-phosphoramidite at 0.15 M in acetonitrile, tetrazole 0.25 M in acetonitrile, 15-20 min. Positions of phosphorothioate linkages are indicated by *; 2'MOE nucleotides are indicated in bold, all C are 5-methyl C; sydnone-modified nucleotides are indicated in blue (ribose modification), in red (nucleobase modification), or in green (phosphate modification).

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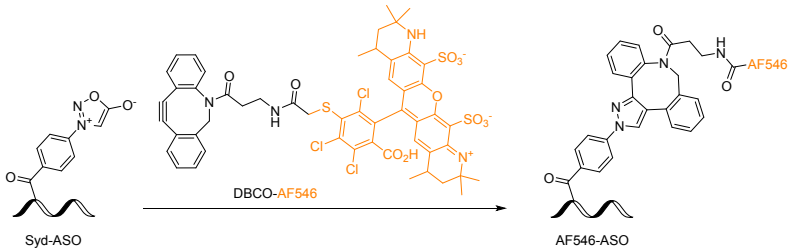

Oligonucleotides	Sequences	DBCO equiv./ reaction time	SPSAC yield
Int-2'-AF546-ASO	**T*G*C*C*T**$^{(2'\text{-AF546})}$***T*T*A*G*G*A*T*T*C*T*A*G*A*C*A**	3.25 equiv./6 h	55%
Ext-2'- AF546-ASO	T$^{(2'\text{-AF546})}$***G*C*C*T*T*T*A*G*G*A*T*T*C*T*A*G*A*C*A**	2.65 equiv./6 h	66%
5- AF546-ASO	**T*G*C*C*T***T*T*A*G*G*A*T$^{(5\text{-AF546})}$***T*C*T*A*G*A*C*A**	2.50 equiv./5h	70%
P- AF546-ASO	**T*G*C*C*T***T*T*A*G*G*A*T$^{(P\text{-AF546})}$***T*C*T*A*G*A*C*A**	2.50 equiv./6 h	64%

Table 2. Post-functionalization of sydnone-modified ASOs and yields of SPSAC reactions. Syd-ASOs (65-85 µM) were treated with 2.5-3.25 equiv. of AF546-DBCO in phosphate buffer (pH 7.5) for 5-6 h at 37 °C and purified by HPLC. Positions of phosphorothioate linkages are indicated by *; 2'MOE nucleotides are indicated in bold, all C are 5-methyl C; functionalized nucleotides are indicated in blue (ribose modification), in red (nucleobase modification), or in green (phosphate modification).

Effect of sydnone modification on ASO-MALAT1 activity and cellular internalization

ASO activity depends on target engagement but first requires productive cellular internalization.[46] To determine whether the sydnone modification influences these two mechanisms, the activities of various sydnone-modified ASOs were examined in human glioblastoma cells (U-87 MG). Two distinct conditions were evaluated: a gymnotic condition to examine the effect of sydnone modification on ASO productive cellular internalization and a condition using a transfection reagent (Lipofectamine RNAiMAX) to eliminate internalization bias and examine the effect of sydnone modification on ASO target engagement. The *MALAT1* lncRNA levels were assessed 48 hours after ASO treatment at different concentrations by RT-qPCR (Figure 2). Under gymnotic conditions, our results show no significant difference in ASO potency between the sydnone-modified ASOs and the non-modified ASO (Ref-ASO) (Figure 2A). In the presence of a transfection reagent, all ASOs demonstrated increased activity, with more than 75% *MALAT1* lncRNA knockdown at the lowest concentration, and showed no significant differences compared to Ref-ASO or among themselves (Figure 2B).

Overall, our results indicate that, under the sequences and experimental conditions investigated in this study, neither the presence nor the position of the sydnone modification appears to significantly affect ASO target engagement. Moreover, the retention of ASO activity under gymnotic conditions suggests that productive cellular internalization remains unaltered, further confirming that sydnone modifications do not compromise ASO function.

Effect of AF546 SPSAC functionalization of sydnone-modified ASOs on their activity and cellular internalization

It has been demonstrated that fluorophores can affect the cellular internalization properties of various molecules.[47] As cellular internalization is crucial for ASO activity, it is necessary to determine whether AF546 functionalization affects both ASO activity and internalization. Consistent with previous experiments, the activities of various functionalized sydnone-modified ASOs were evaluated in U-87 MG cells after a 48-hour ASO treatment at different concentrations, by quantifying *MALAT1* lncRNA levels using RT-qPCR (Figure 2). Under gymnotic conditions, our results show a decrease in the activity of the AF546-functionalized sydnone-modified ASOs, with a significant increase in their AUC (Figure 2C). No difference was noticed among the different AF546 sydnone-modified ASOs. In the presence of a transfection reagent, all ASOs demonstrated increased activity again with similar potency at low concentration as observed previously, and no significant differences compared to Ref-ASO or among the different positions (Figure 2D).

To confirm the impact of AF546 functionalization on the cellular internalization of ASOs under gymnotic conditions, we examined the difference in activity between ASO-AF546 and their sydnone-ASO counterparts at a dose of 1 µM (Figure 2E). AF546-functionalized oligonucleotides consistently displayed lower activity than their non-functionalized counterparts, with statistically significant reductions observed for functionalization at the Ext-2' and 5' positions (p-value < 0.05). This corroborates the influence of the fluorophore on the behavior and internalization of ASOs. Overall, these results indicate that the delivery of sydnone-ASOs into cells before bioconjugation represents a more effective strategy for cellular uptake and subsequent applications.

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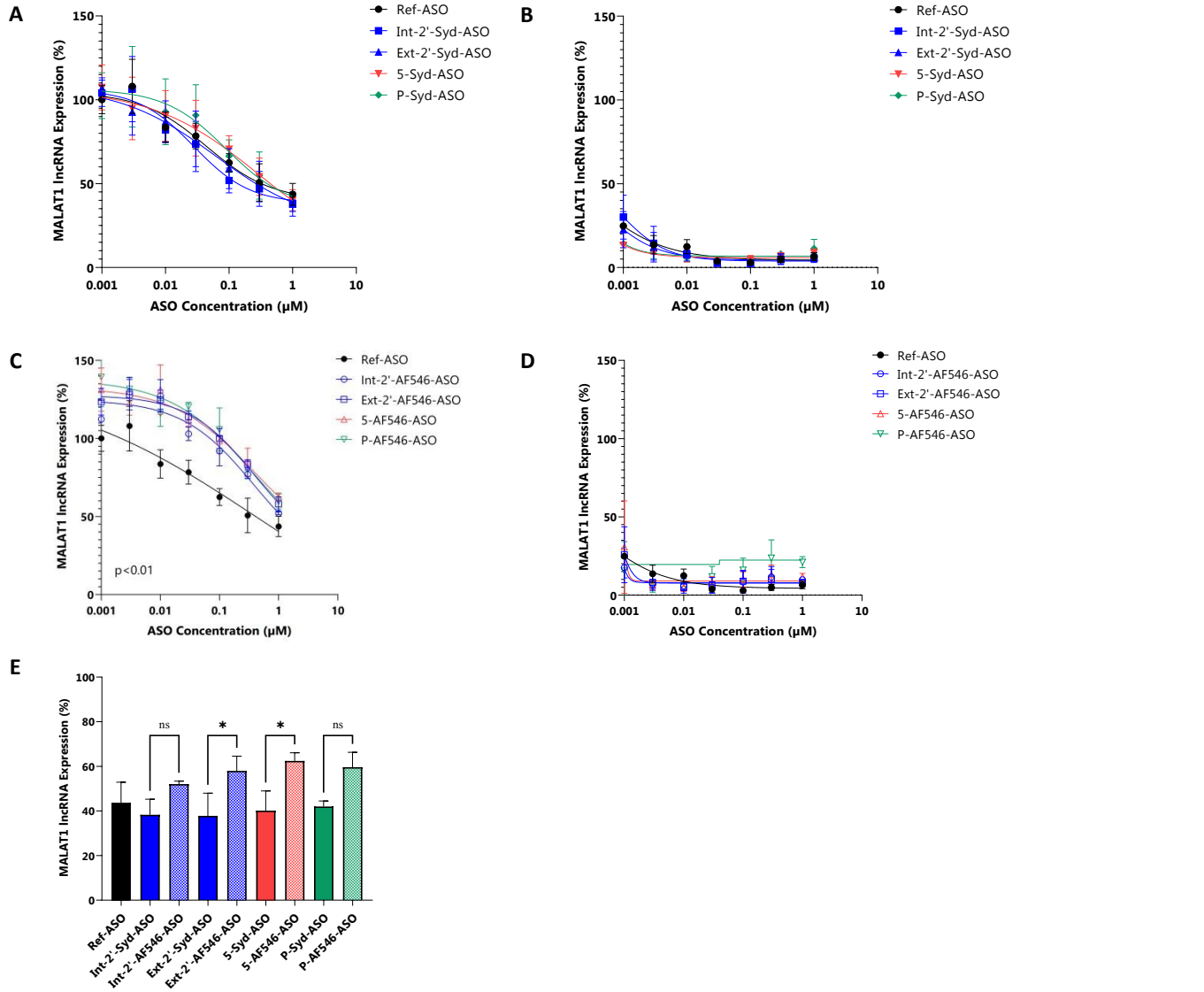

Figure 2. MALAT1 RNA expression analyzed in U-87 MG cells using RT-qPCR. ASOs were transfected with (**B**, **D**) or without Lipofectamine RNAiMAX (**A**, **C**). (**A**) Sydnone-modified ASO activity under gymnotic conditions (**B**) Sydnone-modified ASO activity with a transfection reagent. (**C**) AF546-conjugated ASO activity under gymnotic conditions. (**D**) AF546-conjugated ASO activity with a transfection reagent. (**E**) Comparison of knockdown activity between sydnone-ASOs and their AF546 counterparts. (N = 2 independent experiments, with three technical replicates per experimental data point and sixteen for the control; data are represented as mean ± SEM. GAPDH was used as the reference gene, and an untreated condition served as the control group. Area Under the Curve (AUC) and bars were compared using one-way ANOVA with a p-value < 0.05).

Altogether, these results suggest that AF546 functionalization influences ASO cellular internalization: under gymnotic conditions, AF546-functionalized ASOs show reduced activity despite unaltered target engagement.

Cellular internalization of AF546-conjugated ASOs

To exert their activity, ASOs must enter the cell and engage their intracellular target. To do so, they need to be internalized by cells, mostly through endocytosis.[46] AF546-conjugated ASOs were first evaluated for cellular imaging by confocal microscopy, a widely used method to visualize intracellular ASO localization. U-87 MG cells were treated with different AF546-conjugated ASOs for 48 hours without a transfection reagent. The cells were then fixed and stained at different time points: 30 min, 2 h, 8 h, 24 h and 48 h. The images obtained for the P-AF546-ASO are shown in Figure 3. In the acquired images, no ASO signal was detected in either the untreated condition or the sydnone-modified ASO condition. In contrast, ASOs appeared as distinct spots in the conditions treated with AF546-functionalized sydnone-modified ASOs, with no evidence of channel crosstalk. Furthermore, we observed an increase in detected ASO over time, in the form of intracellular spots. These spots were unevenly distributed and predominantly perinuclear, consistent with ASO trafficking to perinuclear structures such as endosomes.[48]

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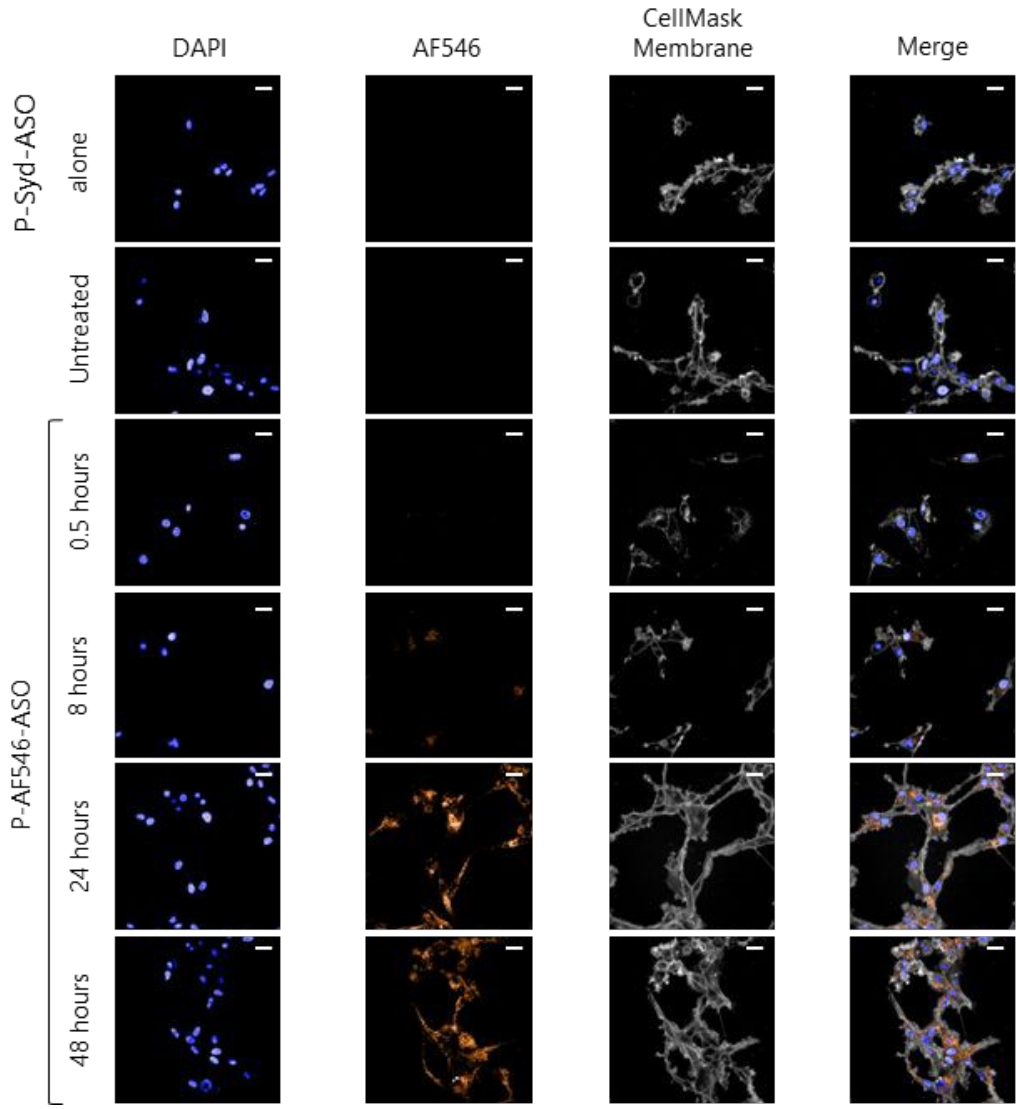

Figure 3. P-Syd-ASO and P-AF546-ASO cellular detection by confocal microscopy in U-87 MG cells treated with 1 µM of ASOs at different time points. The ASOs were not transfected with RNAiMAX. (N = 1 experiment, 60× water-immersion magnification, scale bar = 20 µm).

Hence, our results demonstrate the feasibility of tracking the intracellular distribution of AF546-functionalized sydnone-modified ASOs over time.

Next, we aimed to quantify the observed increase in detected intracellular AF546-ASOs. The images obtained for the Int-2'-AF546-ASO are shown in Figure 4A. By using two metrics, the number of ASO spots detected per cell and their pixel area (Figure 4B), we determined that the number and area of ASO spots increased for the first 24 hours, followed by a decrease in number but a continued increase in area. This observation is consistent with previous reports of ASO intracellular accumulation over time but, to our knowledge, we are the first to describe an increase in their area.[49,50] We hypothesize that most of the detected ASO spots correspond to ASOs contained within intracellular vesicles, as only a small fraction of ASOs can perform endosomal escape.[51] Consequently, the observed decrease in spot number concomitant with an increase in spot area may result from the fusion of these vesicles.

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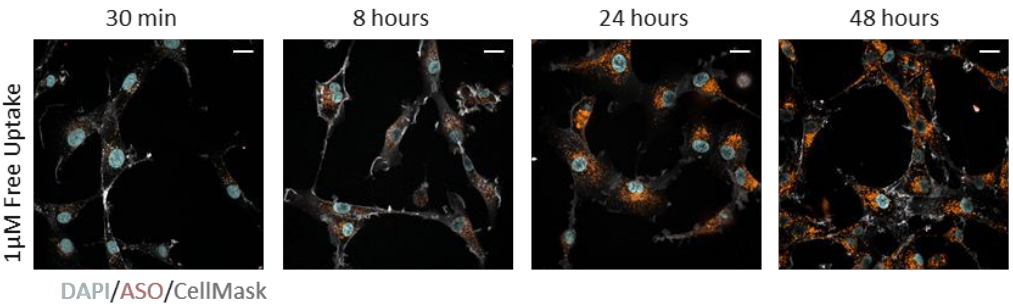

B

Time after ASO transfection:	0.5 h	8 h	24 h	48 h
Number of ASO spots/cell	119.50	149.50	173.00	132.50
Spots Area (px^2)	12.65	15.60	18.35	23.20

Figure 4. Int-2'-AF546-ASO cellular localization: quantification and time-dependent evolution. (**A**) Representative immunofluorescent staining obtained by confocal microscopy of ASO-MALAT1 (Int-2'-AF546-ASO), the nucleus (DAPI) and the cellular membrane (CellMask Deep Red) in U-87 MG cells treated with 1 µM of Int-2'-AF546-ASO for 48 hours under gymnotic conditions (60× water-immersion magnification, scale bar is 20 µm). (**B**) Quantification of sydnone-ASO-AF546 by the number of spots and spot areas. (N = 1 experiment)

Although the presence of the fluorophore on the ASO may introduce some bias, this experiment demonstrates that our tool can be useful to assess ASO cellular uptake in different cell types. Altogether, our results demonstrate that SPSAC-based ASO functionalization provides a novel tool for investigating ASO trafficking and advancing our understanding of this process, as well as an efficient method for bioconjugation to a fluorophore.

Application of in-cell SPSAC conjugation for unbiased ASO cellular internalization studies

As mentioned previously, sydnone-ASOs that are functionalized prior to cellular uptake may be subjected to biases from fluorophore-driven interactions. To overcome this issue, we performed the SPSAC reaction inside cells, following treatment with 5-Syd-ASO both with and without a transfection reagent (Figure 5A and B). HeLa cells were plated and incubated for 24 hours before a 24-hour ASO treatment. Cells were then fixed, washed, and subjected to the SPSAC reaction for 3h using AF546-DBCO. The resulting images showed specific ASO detection, with no signal observed in the condition with AF546-DBCO or sydnone-ASO alone. As expected, the use of the transfection reagent RNAiMAX enhanced cellular ASO uptake, resulting in stronger signals.[52] Nevertheless, signals in the gymnotic conditions remained above background levels. Furthermore, the presence of ASOs was observed within the nuclei, which had not been possible with pre-conjugated ASOs. These preliminary results demonstrate the feasibility of using in-cell SPSAC reactions to detect intracellular ASOs, thereby overcoming fluorophore-related biases. Our tool provides a more reliable method to study ASO cellular uptake, which is crucial for optimizing ASO therapeutics, as a major bottleneck is still their non-productive cellular uptake. Although the SPSAC reaction kinetics with sydnone remain suitable in cell, further optimizations—such as employing more reactive partners like chlorosydnone or fluorosydnone—could significantly enhance the reaction rate.

Conclusions

The therapeutic potential of ASOs is now well established. However, their further development depends on a comprehensive understanding of their intracellular behavior. While ASOs have shown considerable promise in modulating gene expression, their activity depends on a series of complex steps, including cellular uptake, intracellular trafficking, stability, and target engagement. The ability to monitor ASOs within cells therefore represents a major asset for both fundamental studies and translational applications. Upon cellular entry, ASOs encounter multiple barriers, which can significantly limit their availability in the cytoplasm or nucleus, where target engagement occurs. Methods allowing the visualization or quantification of ASOs within specific compartments provide insights to distinguish between passive accumulation and productive delivery to the active site. Furthermore, the correlation between intracellular localization and biological activity is crucial. Monitoring their localization in parallel with functional assays allows for a more accurate interpretation of efficacy data. Moreover, methods to track ASOs contribute to the optimization of chemical modifications and delivery strategies. Backbone, nucleobase or sugar modifications, as well as conjugation to ligands (e.g., lipids, peptides, GalNAc), are widely used to enhance nuclease resistance, pharmacokinetics, and tissue distribution. Tracking ASOs directly in cells provides a powerful means of assessing how such modifications influence cellular uptake, endosomal escape, and subcellular targeting.

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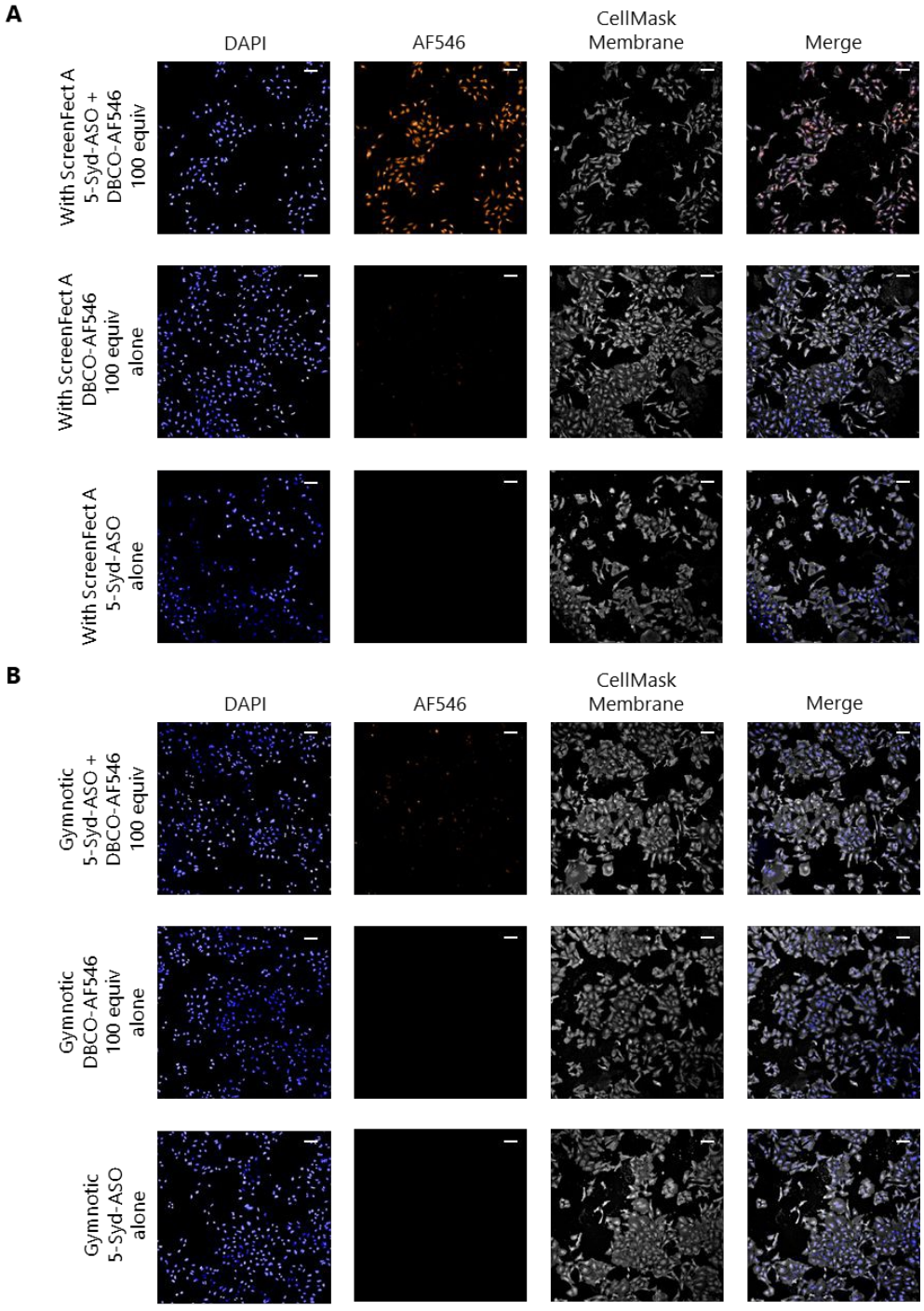

Figure 5. 5-Syd-ASO cellular detection after in-cell SPSAC reaction with confocal microscopy in HeLa cells. ASOs were transfected (**A**) or not (**B**). (10× water-immersion magnification, scale bar ~~is~~ = 20 µm, N = 1 experiment).

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ARTICLE

We have developed a simple and efficient strategy to functionalize therapeutic oligonucleotides via a bioorthogonal SPSAC reaction, providing an attractive alternative to the commonly employed SPAAC methodology. We successfully designed and synthesized three phosphoramidite synthons incorporating the sydnone motif on either the sugar, the phosphate, or the nucleobase core. These building blocks proved to be fully compatible with conventional solid-phase oligonucleotide synthesis, enabling site-specific incorporation of sydnone groups for diverse applications.

At all tested positions, we demonstrated the compatibility of the oligonucleotides with the SPSAC reaction by successfully functionalizing each with a DBCO-conjugated fluorescent probe, thereby validating the versatility of this approach. Importantly, SPSAC offers a valuable alternative to SPAAC in oligonucleotide synthesis, as the incorporation of cyclooctyne groups during automated synthesis can, in some cases, be incompatible with the harsh conditions required.

This bioconjugation strategy can be envisioned for the functionalization of therapeutic oligonucleotides with various functional entities, such as delivery agents, and expands the repertoire of bioorthogonal groups available. This strategy opens new possibilities for multiplexed bioorthogonal reactions, enabling the incorporation of multiple chemical handles within the same oligonucleotide. Indeed, multiple incorporation of bioorthogonal groups that are mutually orthogonal is a promising approach that still requires further developments, particularly in the context of SPOS. It would enable a modular design in which different positions on the oligonucleotide could be independently functionalized through distinct bioorthogonal ligation reactions, such as CuAAC, SPAAC, iEDDA and SPSAC. This would facilitate, for instance, systematic studies of cellular uptake by allowing independent variation of vectorization agents on one hand and labeling tools on the other (e.g., fluorophores, biotin, etc…).

In addition, SPSAC is compatible with biological environments, enabling the functionalization of ASOs after cellular uptake. This post-delivery labeling strategy avoids potential biases arising from altered cell penetration or target engagement. Notably, the sydnone moiety does not interfere with ASO intracellular activity under the conditions tested, underscoring its value as a synthetically robust, minimalist, and versatile motif that may open new opportunities for intracellular ASO chemical modification.

Overall, these developments significantly expand the bioconjugation toolbox for ASO tracking, targeting, and imaging, thereby advancing both their therapeutic applications and our understanding of their intracellular mechanisms.

Author contributions

A.B., R.G. and D.G. designed research; A.B., M.P. and A.K. performed research; F.T. brought expertise in sydnone chemistry; A.B. D.U., A.K. and D.G. analyzed data; D.G. and A.B. wrote the paper.

Conflicts of interest

There are no conflicts to declare.

Data availability

The data supporting this article have been included as part of the Supplementary Information.

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Sydnone-modified nucleosides as versatile tools for bioorthogonal post-synthetic functionalization of antisense oligonucleotides

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