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Efficient synthesis of light-triggered circular antisense oligonucleotides targeting cellular protein expression

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Abstract

Light-activated (“caged”) antisense oligonucleotides are powerful molecules for regulating gene expression at sub-micron spatial resolution, through the focal modulation of endogenous cellular processes. Cyclized caged oligos are particularly promising structures based on their inherent stability and similarity to naturally occurring circular DNA and RNA molecules. Here we introduce an efficient route for cyclizing an antisense oligodeoxynucleotide incorporating a photocleavable linker. Oligo cyclization was achieved for several sequences in nearly quantitative yield through intramolecular copper(I)-catalyzed azide-alkyne cycloaddition (CuAAC). Caging stability and light activation were characterized by FRET efficiency, denaturing gel assay and melting temperature measurement. Finally, a cyclized caged oligo was designed to target *gfap*, which gave 10-fold reduction in GFAP protein upon photoactivation in astrocytes.

Table of Contents

Caged circular oligonucleotides allow photomodulation of gene expression in living cells.

Efficient intramolecular cyclization step improved caged oligo yields.

Keywords

circular oligonucleotide; photo-caged; light activation; antisense; click reaction

Synthetic light-triggered processes provide a useful molecular toolkit for the study and regulation of small, optically transparent biological systems. Light of wavelengths ranging from near-UV to near-IR is orthogonal to many other physical-chemical triggers and when used appropriately does not perturb most cellular processes. The ease of toggling light ‘on’

and ‘off’ and focusing light to a diffraction-limited, sub-micron voxel makes it possible to interrogate the role of spatiotemporal patterning in natural systems. Molecules that are designed to be ‘caged’ have their biological function temporarily blocked by one or more photolabile groups, until light activation restores bioactivity.^[1] Syntheses have been developed for caging many small molecules, but more efficient strategies are still needed for caging larger biomolecules, e.g., oligonucleotides, proteins, and polysaccharides, in order to improve isolable yields and achieve a clean ‘off’ to ‘on’ photo-response.

Light-controlled modulation of gene expression through RNA targeting has been intensely investigated over the past decade. In recent studies, exemplar caged oligonucleotides have been developed, including DNA decoy,^[2] RNA,^[3] siRNA,^[4] antagomir,^[5] analogs such as PNA^[6] and morpholino,^[7] and tested for direct gene knockdown, transcription regulation, and gene editing. RNA targeting, in general, involves binding a complementary oligo to a selected target sequence through highly specific base pairing, which can interrupt downstream processes either by steric hindrance or degradation of the RNA target. Using antisense oligodeoxynucleotides (AS-ODNs) to recruit an endogenous enzyme RNase H is a simple, catalytic approach. The AS-ODN binds to target where RNase H recognizes the AS-ODN-mRNA duplex and degrades the mRNA strand, which prevents translation and releases the AS-ODN to bind subsequent RNA targets.^[8] AS-ODNs have shown great potential in gene function analysis, target identification, and therapeutic development, with three FDA-approved drugs and more than one hundred ongoing clinical trials.^[9] In order to expand applications of AS-ODNs in chemical biology, methods for generating caged oligos in high isolable yield are needed. Notably, the caging method should stably and effectively block biological activity until the AS-ODN is revealed with an efficient photo-trigger.

Three major caged oligo structures have been demonstrated in the literature and explored in our laboratory to achieve the best caging effect. The first incorporates photo-responsive protecting groups on one or several bases. Because binding to target RNA mostly relies on base pairing, base-protecting groups lower the binding affinity, until removed with light.^[10] While solid-phase synthesis incorporating photo-protected nucleotides is convenient and potentially high-yielding, drawbacks are the slow kinetics and possible photo-damage in removing multiple caging groups. A second commonly employed caged oligo structure connects the bioactive oligo to complementary blocking strand(s) via 1 or 2 photocleavable linkers. In this design, the blocking strand(s) should dissociate post-photolysis.^[3, 11] It is important to incorporate the proper length and sequence for both the bioactive oligo and blocking strands in order to achieve both high caging stability and efficient uncaging.^[12] Also, the free blocking strand(s) should not be bioactive. A third caged oligo motif, which avoids multiple caging groups and blocking strands, is a circular structure enforced with a photocleavable linker.^[13] Target binding is inhibited by the curvature of the caged oligo, until photoactivation reveals the bioactive, linear form.

Circular oligos are naturally occurring in living cells, where circularization imparts new functionality, including greater stability.^[14] Synthetic oligonucleotide cyclization has been demonstrated both enzymatically and chemically. In an early example, our laboratory generated a circular caged DNzyme with a photocleavable linker using ssDNA ligase to form phosphodiester linkage between the 3′ and 5′ ends.^[15] Tang et al. developed a

circularization method using amide bond formation.^[16] In a recent paper, a photolabile phosphoramidite linker was synthesized with carboxylate functionality, which was positioned at the 5' end and reacted with 3' amine at rt for 48 h.^[17] The Chen lab synthesized a linker with two reactive moieties that can join the ends of a morpholino modified with amine and disulfide.^[18] Copper(I)-catalyzed azide-alkyne cycloaddition (CuAAC) reaction is another popular choice.^[19] The Seela lab chose a bis-azide linker to react with oligonucleotides terminated with alkynes.^[20] More recently, the Heckel laboratory used bis- and tridentate azido linkers to bend an oligonucleotide containing alkyne groups into two rings and thereby block hybridization.^[21] We note that all of these methods achieved a desired cyclized product, however many of the reactions were laborious, and did not go to completion. This required extensive purification, and isolated yields of circular oligo were typically not reported.

Here we introduce the design of a circular light-activated AS-ODN, which remarkably improved the key cyclization step by employing an intramolecular CuAAC reaction. The caged AS-ODN (Scheme 1) features a circular structure with nitrobenzyl photocleavable linker (marked in purple) connecting the two ends. A short stem facilitated reaction between the ends by pulling them closer together and further stabilized the circular caged state. At the end of the stem, a Cy3-Cy5 FRET pair was incorporated to indicate cellular uptake and caging state. The caged circular oligonucleotide was designed to disfavor binding to target RNA. Irradiation with near-UV light broke the phosphodiester bond between the phosphate and linker (reaction shown in Scheme 1B) and yielded the more-accessible, linear oligonucleotide, capable of targeting mRNA, recruiting RNase H, and catalyzing mRNA degradation.

The RNA target we chose was glial fibrillary acidic protein (GFAP), which is a useful marker of astrocytes encoded by the gene *gfap*. The AS-ODN sequence (Table 1, Sequence 1) was synthesized in three steps. First, a linear strand was synthesized through solid-phase synthesis on 1 μ mol scale, starting with a 3' alkyne CPG, including both fluorophores and bases, and ending with a 5' amine. After cleavage from solid support, the linear strand was purified by RP-HPLC using 0.1 M TEAA and acetonitrile as solvents, which gave 100–200 nmol product. Next, the 5' amine was modified with an azide group using azidobutyrate NHS ester. The reaction was conducted in 0.1 M carbonate/bicarbonate buffer (pH 9) with 200 nmol oligo and 10 $\times$ ester at rt for 2 h. Labelling occurred with 60–70% yield. Both modified product and unlabelled starting material were purified and collected by RP-HPLC. Finally, the modified linear strand was circularized using CuAAC click reaction under optimized conditions.^[22] In Tris-HCl buffer at pH ~7.5, 10 $\times$ Cu(I) was introduced as CuSO₄ reduced by sodium ascorbate in the presence of chelator tris(3-hydroxypropyltriazolylmethyl)amine (THPTA). The cyclization reaction was complete in 1 h at rt and desalted with NAP-5 column (GE Healthcare). Analytical HPLC with peak area integration confirmed generation of the circular product in greater than 90% yield and purity, indicating no HPLC purification was needed for most applications. Comparing HPLC traces in Figure 1A and 1B, the oligo-N₃ peak after 40 min completely disappeared, and the new peak appearing before 30 min was identified by ESI-MS as the desired circular product. ESI mass spectrum in Figure 1B showed the observed mass (7332.4 Da) matched the calculated mass (7333.5 Da). The synthesis was also successfully performed on several additional

sequences: one of similar sequence but with one mismatch in the stem, one with longer stem, and another without fluorophores (Table 1, Sequences 2–4), with HPLC data in Figure S1 and ESI-MS data in Figure S2. Although we and others previously found it necessary to employ excess copper in CuAAC reactions involving oligonucleotides,^[23] it was possible in this case to lower the copper ratio from 10× to 1× to avoid potential toxicity in cellular assays, while achieving cyclized product in similar yields and purity, as shown in Figure S1D.

Caged oligo (sequence 1, Table 1) stability and light activation was initially characterized by FRET efficiency, denaturing gel assay and melting temperature. Photoactivation was performed with a Spectroline transilluminator with 365 nm light, 9 mW/cm² at sample for 15 min. Time course of photoactivation, as monitored by decreasing FRET signal, is shown in Figure S3. To calculate FRET efficiency, fluorescence at 565 nm for Cy3 and 667 nm for Cy5 was measured at 24 °C using 552 nm excitation. After photolysis, Cy5 fluorescence showed a blue shift and similar intensity, while Cy3 fluorescence increased (Figure 2A). To have a better comparison, shown in Figure 2B, FRET was measured for both unreacted oligo-N₃, and cyclized oligonucleotide with or without a complementary strand (“comp”). In the absence of complementary strand, FRET signal increased from 26% to 37% after ‘click reaction’, indicating a structural change bringing the two ends closer. FRET signal decreased to 16% post-photolysis with the complementary strand, which is comparable to the unreacted linear oligo and consistent with complete photoactivation. The significant decrease in FRET signal, consistent with increased distance between the 5′ and 3′ ends, was a useful indicator of uncaging. The modest pre-photolysis FRET signal was consistent with some fraying of the short, 4mer stem at rt. By comparison, in sequence 3 (Table 1) the longer 6mer stem gave a higher initial FRET signal (Figure S4). Photoactivation of caged AS-ODN sequence 1 was confirmed by denaturing gel in Figure 2C, with unreacted linear strand as a control. Without light, the band corresponding to circular oligo ran faster than the linear strand, indicating different secondary structure. After photolysis, the resulting linear oligo migrated slower than the intact circular oligo and reached a similar position as the linear oligo control. After photolysis, melting temperature also decreased from 57 °C to 37 °C (Figure S5). These in vitro characterization data showed that the caged circular AS-ODN can be activated with light.

The caged AS-ODN (sequence 1, Table 1) was transported into astrocytes using PepFect6 (PF6). Developed by Langel and coworkers, PF6 is a cationic peptide that can form nanoparticles with negatively charged DNA.^[24] PF6 incorporates a chloroquine analog to facilitate endosomal escape. To achieve oligonucleotide-PF6 nanoparticles of roughly 100 nm diameter for good cellular uptake (Figure S6), the loading condition was optimized by varying the oligonucleotide concentration, the ratio between PF6 and oligonucleotide, and incubation time. Oligonucleotide and PF6 were premixed in DMEM at rt and diluted to final concentration 0.4 μM and 0.8 μM. Cells were incubated at 37 °C and protected from ambient light after treatment with the complex. Compared to the 0.5 h time point, the average fluorescence intensity roughly doubled after 2 h incubation, indicating uptake (Figure S7). The caged AS-ODN was activated by 405 nm laser showing successful activation with concomitant increase in Cy3 signal and decrease in Cy5 signal (Figure 3).

GFAP protein expression was checked by immunohistochemistry. After loading and photoactivation of oligo-PF6 complex, cells were incubated for three days before fixation to allow time for degradation of GFAP protein expressed prior to oligo loading. Representative image in Figure 4A shows high level of GFAP protein expression in control group with no treatments. A comparable expression level was seen in cells loaded with oligo-PF6 but not irradiated with light (Figure 4C), suggesting the circular AS-ODN remained caged for days. GFAP protein level was reduced approximately 10-fold when the oligonucleotide was loaded and photoactivated (Figure 4B, 4D). The results from immunohistochemistry confirmed that caged circular AS-ODNs of this type can be used to reduce cellular protein levels in a light-dependent fashion.

In summary, a new circular, light-activated AS-ODN was synthesized, characterized, and deployed in astrocytes. We simplified the synthesis by incorporating most of the building blocks on the solid-phase, and conducting an intramolecular CuAAC circularization reaction. The synthesis is compatible with different nucleotide sequences and different stems. This approach has potential for photo-degrading targets such as miRNA or lncRNA. Other changes could also be incorporated to further improve AS-ODN performance. Backbone modification is one way to increase stability and delivery efficiency.^[8a] Progress has also been made in generating photolinkers that can be activated at longer wavelengths, with potential for lower phototoxicity and deeper tissue penetration.^[2, 23b, 25]

Experimental Section

Immunofluorescence:

GFAP expression in astrocytes was analyzed using an Image-iT® Fixation/Permeabilization Kit (Life Technologies) after 3-day treatment with AS-ODN. Briefly, cells were fixed with 4% paraformaldehyde in PBS (pH 7.4) for 15 min at rt. After washing three times with PBS, cells were permeabilized with 0.5% Triton X-100 for 15 min and blocked with 3% BSA for 1 h. Cells were incubated with primary antibody (Abcam, Anti-GFAP antibody, ab16997) diluted 1:100 overnight at 4 °C. Cells were then washed three times with PBS and incubated with secondary antibody (Life Technologies, donkey anti-rabbit IgG, A-31573, 1:1000) for 2 h at rt. The coverslip was mounted on a slide for imaging using VECTASHIELD® Antifade Mounting Medium with DAPI (Vector Laboratories). Imaging was conducted on a Zeiss 710 confocal microscope.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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A) Oligo - azide **B) Oligo - circular** **Figure 1.**

(A) HPLC trace of azide-modified oligonucleotide; (B) HPLC trace of circular AS-ODN product after NAP-5 column, and ESI mass spectrum.

Figure 2.

(A) Fluorescence spectrum of Cy3/Cy5-labelled circular AS-ODN, before and after light activation; (B) Calculated FRET efficiency for linear and circular oligo before and after photolysis, with and without a complementary (“comp”) strand; (C) Denaturing 20% PAGE gel for linear and circular oligo before and after photolysis.

Figure 3.
(A) Photoactivation of AS-ODN in astrocytes. (B) Changes of fluorescence intensity for both Cy3 and Cy5 during photoactivation

Figure 4. Immunohistochemistry selectively imaging GFAP for (A) blank sample without oligo treatment, (B) sample with caged AS-ODN but no photolysis, and (C) sample with caged AS-ODN and photolysis. (D) Quantification of fluorescence staining. Intensity was normalized to the blank sample.

Scheme 1.

(A) General scheme for photoactivation of circular caged oligonucleotide. (B) Detailed structure of circular AS-ODN and reaction of photocleavage

Table 1.

Four different sequences synthesized.

	Sequence	Stem length
1	3' Alkyne Cy3 <u>TCCC</u> AATCAATCAA <u>GGGA</u> Cy5 N ₃ 5'	4-mer
2	3' Alkyne Cy3 <u>TCCC</u> AATCAATCAA <u>GGAA</u> Cy5 N ₃ 5'	one mismatch
3	3' Alkyne Cy3 <u>CGTTGA</u> GGTCAC TTT <u>TCAACG</u> Cy5 N ₃ 5'	6-mer
4	3' Alkyne <u>CCG</u> TTGTAGGACCC <u>CGG</u> N ₃ 5'	3-mer