

Manipulation of gene expression in zebrafish using caged circular morpholino oligomers

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ABSTRACT

Morpholino oligomers (MOs) have been widely used to knock down specific genes in zebrafish, but their constitutive activities limit their experimental applications for studying a gene with multiple functions or within a gene network. We report herein a new design and synthesis of caged circular MOs (caged cMOs) with two ends linked by a photocleavable moiety. These caged cMOs were successfully used to photomodulate β -catenin-2 and *no tail* expression in zebrafish embryos.

INTRODUCTION

Manipulation of a specific gene's function without cross-effects will open exciting opportunities for the study of gene networks. Among many methods, light has stood out as an excellent non-invasive external trigger due to high spatiotemporal resolution. Light-activated (also 'caged') molecules have been used for controlling biological processes for a few decades (1). However, only during recent years have they been widely used in more complex biological applications, such as gene regulation. Photocaged moieties can be used to temporarily 'cage' bioactive molecules whose activities can be restored by light irradiation which removes the caging groups. This method has been used to control gene expression in cells or animals (2–17), and using caged biomolecules, especially, nucleic acids, is one of the most important ways of achieving this control. Photoregulation of antisense oligonucleotides, antigens, siRNAs, microRNAs, DNazymes and RNazymes has been developed for the specific interaction with target genes (18–53). However, it is still challenging to effectively and readily switch gene expression 'on' and/or 'off' and provide high spatiotemporal resolution with caged nucleic acids.

For specific photomodulation of gene expression in zebrafish, several methods have been reported to cage nucleic acids and study their ability to photomodulate gene functions. One method is based on statistical caging

strategy with the attachment of multiple caging moieties to the nucleobases or the backbone of phosphate groups. Ando *et al.* caged a green fluorescent protein (GFP) mRNA and a DNA plasmid through non-specific labeling of the backbone phosphates approximately once every 35 bases with coumarin-based photolabile protecting groups. These caged mRNA and DNA constructs enabled the photoregulation of GFP expression in zebrafish and subsequent study of the role of *lhx2* in zebrafish forebrain growth (49,52). More recently, Deiters *et al.* reported morpholino oligomers (MOs) with multiple photocaged monomeric building blocks and demonstrated the utility of these caged MOs in light-activated control of gene function in zebrafish embryos (26). For the introduction of a single caging moiety, Tang *et al.* (37) applied a heterobifunctional linker to link an antisense strand to a partially complementary sense strand and succeeded in photomodulating gene expression of *chordin* and *bozozok* in zebrafish embryos. Based on the same method, caged MOs were reported to silence the *no tail* (*ntl*) gene by Chen (18,30,38). The rational design of caged MOs showed that caged MOs could be modeled as a competitive thermodynamic equilibrium among caged MO, uncaged MO and uncaged MO/mRNA in *in vivo* environments. More recently, Mayer *et al.* showed their 'Photomorph' to silence GFP expression by using a caged sense RNA to temporarily block antisense MO until light-induced strand break of the sense RNA to release the antisense MO (27).

Caged nucleic acids with multiple caging moieties sterically inhibited the interaction with their targets. However, incomplete uncaging was unavoidable with limited intensity and duration of light irradiation. The employment of a single caging group led to more complete uncaging, but the relative thermostability of the caged and uncaged oligonucleotides, antisense strand/target duplex was difficult to balance, especially, *in vivo* study. The number of paired bases, the positions of paired bases (blunt or staggered), the length of photolinkers, and many other factors all had effect on the photomodulation of gene regulation in zebrafish (30). Uncaged antisense

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oligodeoxynucleotides (ODNs) were not quickly and fully accessible to mRNA binding, and optimized candidates *in vitro* might not be the best one *in vivo* due to complicated environments in biological systems (54). Currently there is still a need for the design of new nucleic acid tools for gene regulation *in vivo*.

A circular ODN may be able to overcome current shortages of caged ODNs with the need of multiple caging groups or a thermodynamic equilibrium due to the application of only a single photolabile linker and the absence of a sense strand. Several photolabile circular oligonucleotides have been reported recently. Richards *et al.* synthesized a photolabile circular DNAzyme enzymatically using T4 ligase and realized photomodulation of RNA digestion (29). Previously, we also succeeded in photomodulating RNA digestion by RNase H through the synthesis of light-activated circular antisense ODNs (24). We sought to apply this circularization strategy to cage MOs for photomodulation of gene expression in zebrafish and expect caged circular MOs (caged cMOs) may be structurally unavailable to stably bind target mRNA [while our manuscript is required for resubmission to include photomodulation of a second gene, a related cMO was recently reported for gene photomodulation in zebrafish online 11 June 2012 (55)]. Here we report this new method to cage antisense MOs (MO-cat2, MO-ntl) by linking two ends of linear MOs with a heterobifunctional photocleavable linker (PL) and study their photomodulation of β -catenin-2 and *ntl* expression in zebrafish embryos. The circularization of the MOs temporarily restricts the opening of MO ring until irradiation, which provides the photocontrol of duplex formation between MO and mRNA. Once light-activation cleaves the chemical bond of PL, uncaged MOs will quickly and completely interact with their target mRNAs without the need for the competition with their sense strands, and the photomodulation of gene regulation will then be readily achieved, as shown in Figure 1.

MATERIALS AND METHODS

General methods

Commercially available MOs with a 5'-end amine on resin and a 3'-end amine (Fmoc protected) were purchased from

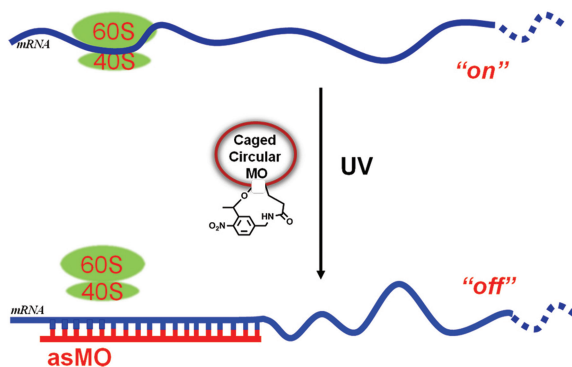


Figure 1. Controlling gene expression using a caged cMO. Photolysis promotes the uncaging of caged cMO and its subsequent binding to mRNA.

Gene-Tools, LLC. All oligodeoxynucleotides were purchased from Sangon Biotech (Shanghai) Co. Ltd. The concentrations of all oligodeoxynucleotides and MOs were measured in water at 260 nm using a Beckman DU 800 Nucleic Acid/Protein Analyzer. All high-performance liquid chromatography (HPLC) was performed on a Varian ProStar 210 equipped with Agilent Zorbax Oligo column or DNAPac PA-200 ion-exchange HPLC column (Dionex, 4 × 250 mm). All mass spectrometric data were obtained using Waters ESI or Shimadzu AXIMA-CFR Plus MALDI-TOF-MS. Zebrafish embryos were injected using microinjector (Harvard Apparatus, PLI-100). All photoirradiation experiments of zebrafish embryos were carried out with UV lamp (365 nm, 11 mW/cm²).

Synthesis of the PL (Supplementary Scheme S1)

An amount of 0.2 g 1-[5-(aminomethyl)-2-nitrophenyl]-ethanol (56) was reacted with ethyl trifluoroacetate (1.2 ml, 10 eq.) in 10 ml ethanol with 0.5 ml triethylamine (TEA) at room temperature overnight. The reaction mixture was concentrated to dryness, and the intermediate was used in the next step without further purification. The dry residue was dissolved in 10 ml acetonitrile, followed by the addition of 0.39 g (1.5 eq.) *N,N'*-Disuccinimidyl carbonate (DSC) and 0.42 ml (3 eq.) dry TEA. The mixture was then stirred at room temperature for 6 h. After removal of acetonitrile, the residue was dissolved in methylene chloride, washed with saturated sodium chloride aqueous solution, and dried with anhydrous Na₂SO₄. The product was isolated using silica gel-column chromatography with ethyl acetate and petroleum ether. The isolated yield was 65% for two steps. ¹H-NMR (400 M, CDCl₃), δ = 1.79 (d, 3H), 2.79 (s, 4H), 4.55–4.80 (m, 2H), 6.32 (m, 1H), 7.48 (d, 1H), 7.62 (s, 1H), 7.83 (s, 1H), 8.07 (d, 1H). ¹³C-NMR (100 M, CDCl₃) 21.5, 25.3, 42.9, 76.45, 125.4, 125.9, 129.0, 137.4, 143.6, 146.5, 150.6, 157.4, 157.8, 169.0 Mass: calculated 433.07, measured: 456.06 (M+Na)⁺, 275.06 (O₂N-C₆H₃-NHCOCF₃).

Synthesis and purification of caged cMOs

To remove Fmoc groups from the protected 3'-amine end of the commercially available MOs, MOs on resin were treated with 20% piperidine in 1,3-Dimethyl-2-Imidazolidinone (DMI) twice and vortexed for 2 min each time, followed by washing with DMI three times and then acetone twice. The obtained resin was suspended in 100 μl DMI solution with PL (1.0 mg, 1.5 μmol) and TEA (1 μL, 6.9 μmol) and vortexed for 24 h. The resin was then washed with DMI three times and acetone twice. The trifluoroacetate protecting group of PL was removed by soaking the resin in cold diluted ammonium hydroxide (0.1 M) for 1 h, followed by washing the resin with water and acetonitrile. Succinic anhydride, *N*-Hydroxy benzotriazole (HOBT) and TEA in DMI were then added to the above dried resin and the mixture was incubated at room temperature for 4 h. The resin was then thoroughly washed with DMI and acetone.

MO was cleaved from resin and deprotected using 0.3 ml of concentrated ammonium hydroxide at 40°C

for 24 h. The solution was chilled for 10 min and centrifuged at 2500 rpm for 5 min. The supernatant was collected, followed by the addition of 10 ml acetone. Precipitated white solid was collected by centrifuging 5 min and further washed with 5 ml acetonitrile. The white solid was dissolved in 1 ml water and the concentration was determined by the measurement of absorbance at 260 nm using a Beckman DU 800 Nucleic Acid/ Protein Analyzer.

Roughly 10 nmol MO after cleavage and deprotection was dissolved in 2 ml 0.1 M TEM buffer with 0.3 M NaCl and 5 mM HOBt. An amount of 1 mg 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDAC) was added to above solution with vortexing. The mixture was shaken at room temperature overnight. To remove the salts, small molecules and small fragments of MO, the reaction solution was passed through a NAP-10 column or was dialyzed against 10% ethanol in water using molecular porous membrane tubing (Spectra/Por Dialysis Membrane, MWCO 3500 from Spectrum, Houston, TX, USA). Concentrated phosphate buffer and acetonitrile were added in accordance with phase A of HPLC purification buffers (20 mM, pH 7.2, 10% acetonitrile). The 2-fold complementary ODN was then added to the above MO solution for further ion-exchange HPLC purification.

For purification of the caged cMO-cat2, Agilent ion-exchange column (Zorbax Oligo) was used. The running conditions for HPLC purification: Phase A, 20 mM phosphate buffer; B, A + 2 M sodium chloride. The following step-wise gradient was used with flow rate of 1 ml/min at 40°C: B, 0% in 10 min, 0–30% in 30 min. The column temperature was maintained at 40°C. The caged cMO was collected in the first 10 min. For purification of the caged cMO-ntl, Dionex Ion-exchange column (DNAPac PA-200) was used. The running conditions for HPLC purification: Phase A, 20 mM phosphate buffer with 10% of acetonitrile; B, A + 1 M sodium chloride. The following step-wise gradient was used with flow rate of 0.9 ml/min at 40°C: B, 0% in 8 min, B, 60–90% in 10 min, B, 90% for 3 min, 0% for 4 min.

Caged cMOs were collected in first 10 min. The unreacted linear MOs formed stable duplexes with their complementary ODN strands and the duplexes moved much more slowly through ion-exchange column due to negative charges of the complementary ODNs. After removing water using a SpeedVac Concentrator, the residue was desalted and concentrated using Millipore-Amicon Ultra-0.5 ml Centrifugal Filters (Cutoff = 3000). The aqueous residue was then freeze-dried to give final white powder. The collected coupling yields of caged cMOs were 20–50% of cleaved MO mixtures after coupling, purification and desalting.

MOs were characterized with ESI-MS (1% TFA in CH₃OH/H₂O 5/95) or MALDI-TOF-MS. Samples were analyzed using a saturated solution of sinapinic acid in 50% acetonitrile/water as a matrix for MALDI measurement. Samples were analyzed according to manufacturer's manual. MO-cat2: before cyclization, 9036.0; after cyclization, 9020.0; MO-ntl: before cyclization, 9117.7; after cyclization, 9099.5.

Fluorescence measurement of molecular beacon with caged cMO-ntl

A complementary ODN molecular beacon (MB-30: 5'-6-Fam-ATCGGAAATATGCCTGCCTCAAGTCCC GAT-Dabcyl-3', HPLC purified) was used for the fluorescence binding assay experiment. The concentrations of stock solutions of MB-30, native MO-ntl, caged cMO-ntl in ultra-pure water were determined by the absorbance at 260 nm. The final concentration in 1× PBS buffer (SIGMA) of molecular beacon was 100 nM, while the concentrations of linear MO-ntl and caged cMO-ntl were 200 nM. To prepare uncaged MO-ntl solution, caged circular MO-ntl solution was irradiated using a UV lamp (365 nm, 11 mW/cm²) for 10 min. After a short vortex, the above solutions were incubated for 5 min at 37°C. Fluorescence spectra were measured immediately with a Cary Eclipse fluorescence spectrophotometer with the excitation wavelength at 488 nm at 37°C.

Gel-shift assay for evaluation of MO/target binding

To determine the binding of a complementary oligodeoxynucleotide to caged cMO-ntl before and after light irradiation, gel mobility shift assays were performed. An amount of 100 nM ODN-38 (5'-ACATTTCGATCGGAA ATATGCCTGCCTCAAGTCTGTACT-3') was added to 200 nM linear, caged circular and uncaged MOs in 20 mM phosphate buffer (pH 7.2). The ODN/MO mixtures were annealed by incubation at 37°C for 30 min and then were put into ice water. One microliter DNA loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF, 30% glycerol in water) was then added to above solutions. All solutions were loaded into 15% native polyacrylamide gels. The gels were then electrophoresed at 100 V for 2 h using 1× TBE buffer (pH 8.2). Gels were exposed and incubated in 1× SYBR Gold nucleic acid gel stain solution for 20 min. The images were acquired by Bio-rad ChemiDoc XRS system.

Zebrafish embryo injection and phenotype observation

Embryos bred from wild-type zebrafish were collected at one-cell stage. Linear and caged cMOs were each injected into zebrafish embryos (1 mM, 5 nl for MO-cat2 and 25 μM, 5 nl for MO-ntl) at one- or two-cell stage. The embryos were then put into an incubator at 29°C. At 3 hpf, half of embryos injected with caged cMO were irradiated with 365-nm light (11 mW/cm²) for 10 min. The irradiated embryos were then put back to the incubator. At 24 hours post-fertilization (hpf), zebrafish phenotypes were observed. The embryos were kept in the incubator for another 7 days and the survived zebrafish was imaged again.

RESULTS AND DISCUSSION

Synthesis and purification of caged cMOs

Synthesis of caged cMOs started with MOs on resin, as shown in Figure 2. In order to introduce primary amine and carboxylic acid at two ends of MOs, the custom-synthesized MOs with Fmoc-protected amine at

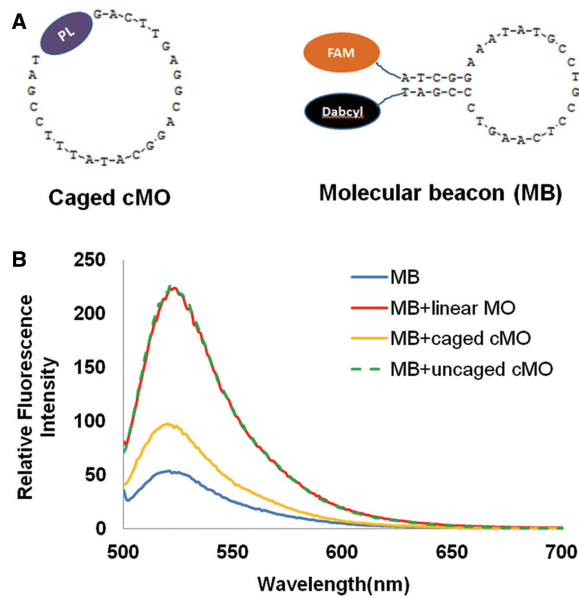


Figure 4. (A) Structures of caged cMO-ntl and its complementary molecular beacon (MB) with FAM and Dabcyl; (B) Relative fluorescence intensity of MB, MB + linear MO-ntl, MB + caged cMO-ntl and MB + caged cMO-ntl + 10 min irradiation at a concentration of 100 nM MB in PBS buffer at 37°C, λ_{ex} = 488 nm.

development. Embryos with 10 min irradiation and no injection developed normally as shown in Figure 5A and B for the sideview and topview. As a positive control, we injected zebrafish embryos with 1 mM linear MO-cat2 (total embryos, $n = 80$) and these embryos developed clear morphogenesis of missing β -catenin-2 at 24 hpf as shown in Figure 5C and D. For embryos injected with 1 mM caged cMO-cat2 (total embryos, $n = 204$) targeting β -catenin-2 at one-cell or two-cell stage, half of the embryos were subject to global irradiation after 3 h post-fertilization (hpf), and the other half were still covered with aluminum foil to avoid light irradiation. As we expected, 91% embryos with caged cMO-cat2 injection had the same normal development as the uninjected ones if the embryos were kept in dark (Figure 5E and F). However, light activation triggered uncaging of the caged cMO-cat2 and promoted its binding to target β -catenin-2 mRNA. Of irradiated embryos, 93% showed block-shaped somites and/or pin head (Figure 5G and H). These phenotypes were identical to the embryos injected with the linear MO-cat2. Light irradiation by itself under our conditions did not show any toxic effects on the development of zebrafish embryos, so the phenotypes can be attributed to the uncaged MO-cat2.

To further verify long-term effect of caged cMO and uncaged MO on zebrafish development, embryos were kept in an incubator for another 7 days and were imaged again. Without injection, the fish showed exactly normal development. Whereas injection with the linear MO-cat2 caused zebrafish to show narrow head and/or much shorter tail (loss of notochord) which were the typical phenotypes for β -catenin-2 defective mutants. Zebrafish embryos injected with the caged cMO-cat2 at one-cell or two-cell stage showed the same normal development as

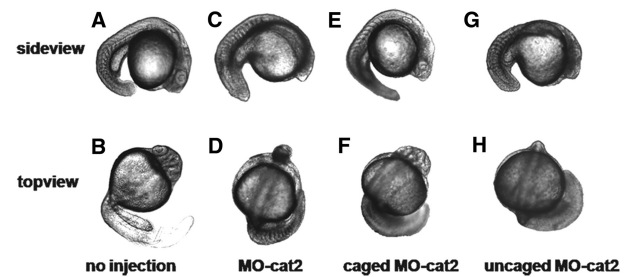


Figure 5. Transmitted light images of representative 24-hpf zebrafish embryos with side view and top view. (A, B) Uninjected embryos irradiated for 10 min developed normally. (C, D) Embryos with 1 mM linear MO-cat2 injection exhibited clear β -catenin-2 defective phenotype. (E, F) Injection with 1 mM caged cMO-cat2 had no effect in the dark. (G, H) Injection with 1 mM caged cMO-cat2 and 10-min UV-irradiation at 3 hpf gave β -catenin-2 defective phenotype.

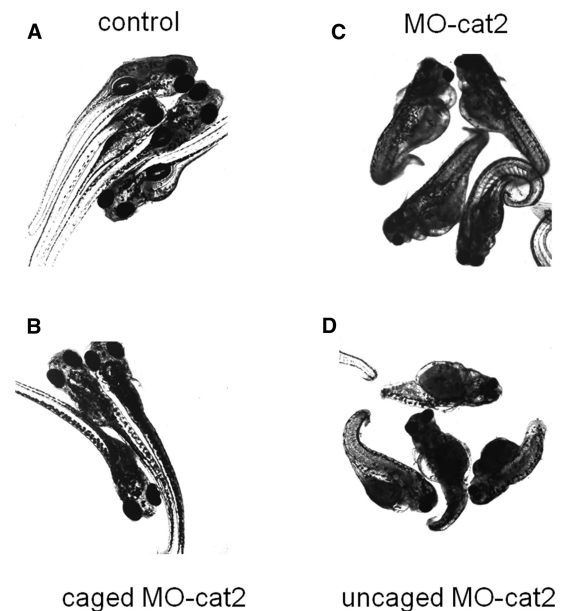


Figure 6. Transmitted light images of representative 8-day zebrafish embryos. (A) Uninjected embryos UV-irradiated at 3 hpf for 10 min developed normally. (B) Injection with 1 mM caged cMO-cat2 had no effect on embryos in the dark. (C) Embryos injected with 1 mM the linear MO-cat2 exhibited a clear β -catenin-2 defective phenotype. (D) Injection with 1 mM caged cMO-cat2 and 10-min UV-irradiation at 3 hpf gave no- β -catenin-2 phenotype of embryos.

the uninjected embryos if no light was applied. However, embryos injected with the caged cMO-cat2 and irradiated with 365-nm light showed the clear phenotypes which were similar to the phenotypes of embryos injected with the linear MO-cat2, as shown in Figure 6. These results indicate embryos injected with caged cMO and uncaged MO maintained their phenotypes for quite long time.

Photoregulation of ntl gene expression in zebrafish embryos using caged cMO-ntl

To test the specificity of a caged cMO, we targeted a second gene, *ntl*, which plays an important role in nodal-independent tail somite formation. The sequence (MO-ntl, 5'-GACTGAGCAGCAATTCCGT-3') has been

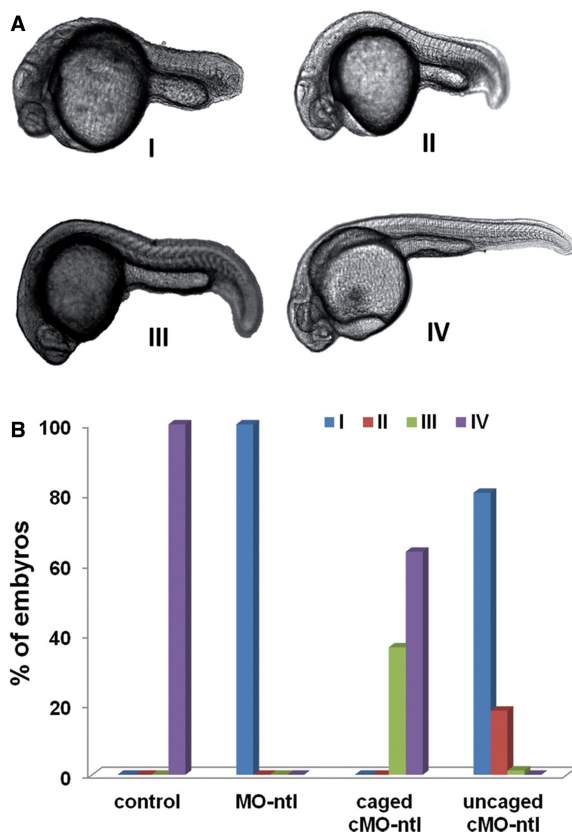


Figure 7. (A) Transmitted light images of representative zebrafish embryos with different class of *ntl* phenotypes. (B) Phenotypic distributions for embryos injected with the indicated linear MO-ntl or caged cMO-ntl and incubated in dark or irradiated with UV light at 3 hpf.

proved effective in knockdown of *ntl* gene expression and resulted in the obvious *ntl* phenotypes with missing notochord cells. Zebrafish embryos are sensitive to the dose of MO-ntl which causes different phenotypes that can be categorized into four classes according to their severity (Figure 7A). Light irradiation by itself did not cause the *ntl* phenotype under our experimental conditions. However, when embryos were injected with linear MO-ntl (125 fmol/embryo), all the embryos (total embryos, $n = 72$) developed into class I phenotype as shown in Supplementary Figure S4 and Figure 7B. Embryos injected with the same amount of caged cMO-ntl ($n = 170$) was divided into two dishes. One dish was subject to global light irradiation at 3 hpf, while the other one was still covered with aluminum foil to avoid light irradiation. As seen in Figure 7B, all the embryos with caged cMO-ntl injection and 10-min irradiation show obvious *ntl* phenotypes predominantly in class I and class II. While the majority of these injected most embryos covered with aluminum foil developed into the same normal phenotypes class IV as the uninjected embryos (Figure 7B). Some class III phenotype embryos were also observed. This may be due to light exposure of caged cMO-ntl during time period of MO injection. Since zebrafish embryos are sensitive to this MO-ntl sequence, even a small amount of uncaged MO-ntl could lead to the observation of a weak *ntl* phenotype as indicated in Figure 7B.

CONCLUSION

We designed and synthesized a new kind of caged MOs with the circular structure. The synthesized caged cMOs were purified with ion-exchange HPLC through a complementary ODN-assisted method. The binding ability of caged cMOs and their target sequences was further characterized with HPLC, fluorescence emission and native PAGE gel, which indicated caged cMOs are immune to target binding. However, they are immediately and fully accessible to target binding upon light activation, which overcomes the previous shortages due to multiple caging groups or relative thermostability of a three-component system. By injecting caged cMO-cat2 and MO-ntl into zebrafish embryos, gene expression was efficiently photomodulated, and clear phenotypes of missing β -catenin-2 and *ntl* gene expression were observed upon light activation during development of zebrafish embryos in both 24 h and several days after fertilization. This new kind of caged cMOs will be promising for the practical applications to spatiotemporal photoregulation of gene expression.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online: Supplementary Figures 1–5 and Supplementary Scheme S1.

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Conflict of interest statement. None declared.

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