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Caspase-Activated Oligonucleotide Probe

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Abstract

Light-activated (“caged”) oligonucleotides provide a strategy for modulating the activity of antisense oligos, siRNA, miRNA, aptamers, DNazymes, and mRNA-capturing probes with high spatiotemporal resolution. However, the near-UV and visible wavelengths that promote these bond-breaking reactions poorly penetrate living tissue, which limits some biological applications. To address this issue, we describe the first example of a protease-activated oligonucleotide, capable of reporting on caspase-3 during cellular apoptosis. The 2'-F RNA-peptide substrate-peptide nucleic acid (PNA) hairpin structure was generated in 30% yield in a single bioconjugation step.

Graphical Abstract

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ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.bioconjugchem.0c00362>.

Supplemental figures for DLS measurement, cell uptake, and comparison with CellEvent (PDF)

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Methods for automated solid-phase synthesis of oligonucleotides have led to the development of diagnostics and therapeutics that are revolutionizing Biology and Medicine.^{1,2} Conditionally activated (‘caged’) oligonucleotides enable spatiotemporal regulation of oligo function, e.g., for controlling in vivo gene expression. Typical caged molecules remain biologically inert until activated with near-UV or visible light. Many examples of light-activated oligos have been developed in recent years, including caged antisense oligos,^{3,4} siRNA,^{5,6} miRNA,⁷ aptamers,⁸ CRISPR-Cas9,^{9,10} and DNAzymes.¹¹ Several versions of light-activated morpholino antisense oligonucleotides have been developed to modulate gene expression in living zebrafish embryos.^{12–14} In addition, our laboratories have developed transcriptome in vivo analysis (TIVA) technology, whereby caged oligos permeate living brain tissue and allow photochemical capture and isolation of mRNA from single neurons.^{15,16} Such approaches provide unique capabilities in thin tissue sections and optically transparent model organisms but are fundamentally limited by poor light penetration, particularly with activation at near-UV wavelengths.

Notably, naturally occurring processes employ enzymes to regulate structure, function, and localization of biomolecules at all time and length scales, e.g., via modifications of DNA, RNA, protein, lipid, and metal ion content, as well as cell, tissue, and organ remodeling, including bone. Enzyme-activated molecules have been widely developed as pro-drugs,^{17,18} for controlling cellular delivery¹⁹ and generating a “turn on” signal in diagnostic imaging.²⁰ However, enzyme-activated oligonucleotides have received scant attention. This new class of caged oligo presents significant challenges in all aspects of design, synthesis, characterization, and biological application. In a singular example, the Chen lab demonstrated an *Escherichia coli* nitroreductase NfsB-activatable morpholino antisense oligonucleotide. A morpholino oligo targeting *no tail-a* (*ntla*) gene was caged via cyclization with a bifunctional NfsB-sensitive 4-nitrobenzyl linker, which was synthesized in 6 steps. The cyclic oligo was linearized and became active in zebrafish embryos injected with NfsB mRNA, which resulted in significant loss of Ntla protein.²¹ Here, we present the first

example of a protease-activated oligonucleotide, which was generated in a single bioconjugation step and serves as a functional reporter of caspase-3 in cellular apoptosis.

Apoptosis is a type of programmed cell death, which plays an important role in maintaining homeostasis in multicellular organisms. Dysregulation of apoptosis is related to several diseases: in tumor cells apoptosis is inhibited leading to unchecked growth and proliferation,²² whereas in neuro-degenerative diseases increased apoptosis causes excessive cell loss.²³ The caspase enzymes are a family of cysteine proteases that serve different functions in cell death and inflammation. Mammalian caspases can be classified as initiator caspases (caspase 2, 8, 9, and 10), executioner caspases (caspase 3, 6, and 7), and inflammatory caspases (caspase 1, 4, 5, 11, and 12). The complex mechanisms of apoptosis are regulated by a cascade of caspases through different signaling pathways, but all routes converge at caspase-3 activation.²⁴ The caspase-3 executioner enzyme typically exists as inactive zymogen or pro-enzyme in cells. When activated, caspase-3 is first proteolyzed into two fragments and then dimerizes into its active form. The activation of caspase-3 starts the process of cell death.^{25,26} We set out to develop a prototypical enzyme-activated oligonucleotide whose binding to target RNA could be initiated by the enzymatic activity of caspase-3.

RESULTS

Design and Synthesis.

The structure of the enzyme-activated oligo is shown in Figure 1. It features a hairpin stem-loop structure with a 14mer 2'-F RNA CG-terminated poly-U capture strand at the 3' end connected to a 13mer blocking strand, which includes an 8mer peptide nucleic acid (PNA)–with terminal GC for efficient base-pairing and 6mer poly-A– and a 5mer caspase-3 peptide substrate. The melting temperature of DNA could be estimated from Marmur-Doty formula, $T_m = (nA + nT) * 2 + (nG + nC) * 4$, where nA, nT, nG, and nC are the number of each nucleotide.²⁷ The 2'-F modification was observed previously to increase the binding affinity for RNA target and raise T_m by as much as 2 °C per residue,²⁸ which allowed use of a short sequence as the capture strand. The probe also includes a 3' biotin moiety for affinity purification and a Cy3-Cy5 FRET pair to indicate cell uptake and enzyme activation. The stem GC pair was installed to align the two strands and achieve good caging and high FRET efficiency, which was also validated in our recent TIVA probe designs.¹⁵ Here, the probe is designed such that in cells expressing caspase-3, the DEVDK peptide substrate is accessible for cleavage. To demonstrate the potential for harvesting mRNA in TIVA applications, the short PNA blocking strand should dissociate postproteolysis, and the released poly-U strand should bind to a target poly-A tail of mRNA. The bound mRNA could be further isolated and purified by high-affinity biotin–streptavidin interaction.

The designed probe was successfully synthesized as two separate strands and only required one final bioconjugation step to generate the caged hairpin stem-loop structure (Figure 1b). First, the 14mer 2'-F-RNA CG-Poly-U capture strand was made on an ABI 394 synthesizer using solid-phase synthesis with phosphoramidites from Glen Research. The synthesis started from a 3'-biotin labeled CPG and finished with alkyne modifier at the 5' end. The product was purified by RP-HPLC using a C18 column. 200–250 nmol product was

collected from 1 μ mol scale synthesis. The 13mer peptide nucleic acid (PNA)–peptide conjugate, which includes a GC-poly-A oligo blocking strand and the 5mer peptide substrate, was ordered from PNA Bio. Caspases specifically recognize 4-residue peptide substrates P4–P3–P2–P1↓P1', with a strong preference for Asp on the P1 position right before the cleavage site (↓) as well as a weak tolerance for charged residues at the P1' position after the cleavage site.^{29–31} The peptide sequence D-E-V-D is the canonical substrate of caspase-3.³² An additional K was included with an azido moiety on the side chain replacing the original amine for subsequent bioconjugation reaction.

PNA is an oligomer with various purine and pyrimidine nucleobases attached with a polyamide backbone. The PNA pseudopeptide structure makes it easy to generate PNA–peptide conjugates in the same solid-phase synthesis. A benefit of using PNA is the neutral backbone, which reduces the electrostatic repulsion with a complementary oligo sequence. This improves the yield in the conjugation step between PNA–peptide and oligonucleotide. In addition, due to the high binding energy of a PNA–oligonucleotide duplex,³³ the hairpin structure remains stable with fewer base pairs.

The PNA–peptide and oligonucleotide were conjugated by so-called CuAAC reaction (Cu-mediated azide–alkyne cyclo-addition)^{34,35} using optimized conditions. The reaction was done in Tris-HCl buffer at pH = 7.5 with 200 mM NaCl. A high concentration of salt was used to counterbalance the negative charges from aspartic acid and glutamic acid on the peptide and the phosphate groups on the RNA capture strand. Excess Cu(I) (10 $\times$) was introduced as CuSO₄ reduced by sodium ascorbate in the presence of the chelator tris(3-hydroxypropyltriazolylmethyl)amine (THPTA). The final concentration of PNA blocking strand was 0.1 mM with 4 $\times$ capture strand. The mixed solution was blanketed with N₂ and reacted at rt for ~14 h.

RP-HPLC purification was done at 60 °C, using TEAA and acetonitrile as solvent. This high temperature was used to melt secondary structure for a better separation. Shown in Figure 2a, the product eluted at around 29 min with absorbance at 254, 552, and 643 nm confirming the conjugate had both Cy3 and Cy5 attached. Excess capture strand only showing signal at 254 and 552 nm was also collected at ~26 min and stored. Final isolated yield of the desired conjugate was 30% based on the quantity of starting PNA measured from absorption at 260 nm. The identity of the desired product was confirmed by ESI mass spectrometry (Novatia, LLC) showing an observed mass of 9009.7 Da in good agreement with the calculated mass (9010.6 Da) shown in Figure 2b. The purity of the bioconjugate was confirmed by analytical RP-HPLC (Figure S1).

Caging Stability and Enzyme Activation.

Oligo hairpin stability and caspase-3 activation were tested through FRET measurement and denaturing gel assay. In Figure 3a, FRET efficiency was first measured at rt, after all samples at 1 μ M were incubated in enzyme assay buffer at 37 °C for 4.5 h with or without caspase-3 and target poly-A strand. The FRET efficiency was calculated using the formula, $\text{FRET} = I_a / (I_a + (I_d \times \gamma))$, where I_a was the emission intensity of Cy5 acceptor at 667 nm and I_d was Cy3 donor intensity at 565 nm using 552 nm excitation; $\gamma = 2$ was the correction of quantum yields for the two fluorophores. The caged probe alone showed a high FRET

signal of 71.2%, which is comparable with an earlier caged oligo hairpin structure.^{15,16} This FRET signal was not influenced by poly-A addition, indicating it was stably caged. When incubated with caspase-3, the FRET efficiency decreased to 30.0% after 4.5 h. The FRET efficiency lowered even further to 21.7% when poly-A was in the solution indicating the blocking strand was displaced by the target and diffused away. Repeating the measurement at different temperatures from 15 to 90 °C gave an estimation of melting temperature. Shown in Figure 3b, before enzyme activation, the probe had a high T_m of 69 °C, consistent with the fully caged structure. The structure was much less stable after enzyme activation: T_m decreased to 34 °C. A large T_m of –35 °C also underscores the success of the caging strategy. Denaturing gel in Figure 3c presented a similar result. The probe incubated in buffer without caspase-3 showed only one band (lane 3) with fluorescence from both Cy dyes. The intact probe migrated similarly to the smaller PNA strand (lane 1), as negative charges from the attached RNA strand promoted electro-phoresis of the probe. After incubation with enzyme, PNA–peptide strand lost the terminal K(N₃) which led to a higher mobility in lane 2 compared to lane 1. Likewise, the K(N₃) was transferred from PNA–peptide strand to the poly-U capture strand for the conjugated probe (lanes 4 and 5). The capture strand showed Cy3-oligo signal running lower and blocking strand with Cy5 signal running higher on the gel. As enzyme concentration was increased from 0.5U to 2U, Cy3 signal decreased in the upper band, indicating almost complete cleavage by caspase-3.

Cell Delivery and *in Vivo* Activation.

Stem-loop oligo hairpins are often delivered to cells using cell penetrating peptides (CPPs) that are noncovalently associated via electrostatic and hydrophobic interactions.^{36,37} We chose Pep-3, a sequence optimized for PNA that can form stable nanoparticles with oligos and facilitate cellular uptake.^{38,39} To achieve a 100–300 nm size range that was suitable for cell uptake, we tuned the ratio between CPP and oligo hairpin and monitored the particle diameter with dynamic light scattering (DLS). Generally, the particle size decreased with a decreasing ratio of peptide to oligo, but no particles were observed when this ratio was too low. Pep-3 in 2.5:1 ratio with oligo made stable particles measured by DLS to be 260 ± 5 nm (Figure S2).

The oligo probe was tested for uptake by HeLa cells, visualized by confocal laser scanning microscopy. Images of both Cy3 and Cy5 fluorescence were collected using a 543 nm He–Ne laser. After 3 h of oligo incubation, high FRET signal suggested the probe was taken into cells and remained caged (Figure S3). Apoptosis was triggered with staurosporine (STS) and confirmed after 7 h by a commercially available probe CellEvent (Figure S4), which features the same DEVD substrate conjugated to a fluorogenic dye. After 8 h STS treatment, an increase in Cy3 signal and a decrease in FRET (37.6%) was observed consistent with probe activation (Figure 4) whereas the control experiment without STS retained high FRET (66.4%) at the same time point. Cy5 intensity was also slightly higher in STS-treated cells, likely due to increased membrane permeability resulting from apoptosis.⁴⁰ As reported in the literature, caspase-3 in pro-enzyme form is predominantly located in the cytoplasm, and the active form can be translocated to the nucleus.^{41,42} Here we observed Cy3 signal in both cytoplasm and nucleus, suggesting activation of the oligo probe in both cellular regions.

DISCUSSION

We have introduced the first example of a caged oligonucleotide activated by a protease, in this case caspase-3. The synthesis included generating a capture strand and blocking strand with peptide linker separately and then joining the two strands in a 1-step bioconjugation reaction. A variety of methods, such as CuAAC,^{43,44} strain-promoted azide-alkyne cycloaddition (SPAAC),^{45,46} thiol-maleimide reaction,⁴⁷ and reductive amination,⁴⁸ have been developed for oligo-peptide conjugation. Oligo-peptide conjugates have been applied to improve delivery efficiency⁴⁹ or as biomaterials.⁵⁰ Neutral or positively charged oligonucleotides are commonly used in these cases. However, in our initial attempts to synthesize a caspase-3-activated oligo probe, we found it difficult to join a negatively charged (DEVD-containing) peptide with a negatively charged oligonucleotide through CuAAC, SPAAC, or thiol-maleimide reactions. To overcome this issue, we applied an 8mer PNA oligo blocking strand with 5mer peptide substrate synthesized altogether. Prehybridization of PNA and RNA strands likely increased the effective concentration of the reactants for CuAAC and promoted this reaction. Other factors such as oligo concentration, salt concentration, ratio of the two strands, nitrogen blanket, and purification conditions also contributed to the acceptable 30% final yield.

Robust stability, while still poised for ready activation, is an important feature for caged molecules. By incorporating a FRET pair, we were able to observe both caging and activation via FRET efficiency, T_m measurement, and denaturing gel assay. A high FRET efficiency for the probe in the presence of poly-A RNA indicated that the capture and blocking strands were well aligned with each other, and the hairpin structure was not opened by the target alone. Once incubated with caspase-3, the probe was activated for RNA target binding, as indicated by all three characterization methods.

Efficient cell delivery is the first obstacle for *in vivo* study. Most commercially available transfection reagents are engineered toward molecules with a certain size and structure. For a probe containing three different backbone structures, we tested a specific CPP under optimized conditions and observed cell delivery and enzyme activation during apoptosis. One potential application of this enzyme-activated oligo would be to harvest mRNA from cells undergoing apoptosis. However, full-length mRNA isolation is challenging in this case. As caspase-3 is a late-stage enzyme in the apoptotic cascade, it took several hours for caspase-3 to be upregulated after STS was introduced and additional time for the oligo probe to be activated. Meanwhile, the transcriptome has been reported to be degraded on the same time scale during apoptosis.⁵¹ However, if a poly dU sequence is the capture strand, even if the RNA is degraded, that fraction that still has a poly-A tail, even if not full-length, could still be isolated and characterized. These experiments will be forthcoming.

We have shown a new caging strategy where a peptide substrate is incorporated within a hairpin stem-loop oligonucleotide. Conjugation yield of 30% was achieved and should be possible to increase further, particularly for nonanionic peptide substrates. This approach complements conventional caging methods in cases where light activation is not practical. DEVD peptide substrate used both in our oligo probe and in the commercially available CellEvent reagent required several hours to be cleaved in HeLa cells undergoing apoptosis.

Further improvements could be made on the specificity of the substrate and to improve the kinetics of proteolysis.^{52,53} Furthermore, this probe design is readily transferable to other protease substrates. The 2'-F RNA capture strand could be replaced with an antisense DNA oligo to achieve biologically controlled antisense gene modulation under a specific physiologic condition.

METHODS

Synthesis of the poly-U Capture Strand.

The capture strand was synthesized on ABI 394 synthesizer using established solid-phase phosphoramidite chemistry.¹⁵ After the synthesis, the oligo was manually cleaved from the CPG support with 30% ammonium hydroxide overnight on the benchtop.

Conjugation of Capture and Blocking Strands.

A 3 nmol reaction was conducted in Tris-HCl buffer (pH = 7.5) with 200 mM NaCl final concentration in overall reaction volume of 30 μ L. Three nmol PNA blocking strand (PNA Bio) and 12 nmol RNA capture strand were added to premixed 10 $\times$ of copper sulfate and 100 $\times$ of THPTA ligand. Sodium ascorbate (80 $\times$) reducing agent was added at last. The oxygen in the system was carefully removed through purge-refill cycle with nitrogen. The reaction was left at rt for 14–16 h. The final product was purified through RP-HPLC and stored in 1 $\times$ STE buffer (pH = 8) at –20 °C.

FRET Measurement and T_m Calculation.

A final concentration of 0.5 μ M probe was diluted in enzyme assay buffer (20 mM PIPES, 100 mM NaCl, 10 mM DTT, 1 mM EDTA, 0.1% CHAPS, 10% sucrose, pH 6.5) to a sample volume of 80 μ L. 1 $\times$ of 30mer poly-A RNA as a model poly-A tail was added to “+poly-A” samples. 0.2 U (1U: Cleave 1 mmol/h at 37 °C) of human caspase-3 (Enzo Life Sciences, Inc.) was added to “+caspase-3” samples. All samples were incubated at 37 °C for 4.5 h. FRET was measured on a Cary Eclipse fluorimeter (Varian) with Cary Temperature Controller (Agilent) at 37 °C. The excitation was 552 nm, and the emission spectrum was collected, 555–705 nm.

For T_m measurement, the same FRET measurement was done at every 0.5 °C from 10–85 °C at a ramp rate of 1 °C per minute. The T_m was determined based on the first derivative of FRET over temperature.

Denaturing Gel.

Each sample was prepared with a 10 pmol probe in 1 $\times$ STE buffer (pH = 8) to a sample volume of 5 μ L and incubated at 37 °C for 4.5 h. The assay was run on 20% PAGE gel with 7 M urea at 200 V for 1 h.

Cellular Uptake and Apoptosis.

2.5 $\times$ of Pep-3 (Thermo Fisher Scientific) was sonicated for 10 min and incubated with oligo at 37 °C for 25 min. HeLa cells were plated on an 8-well coverslip (ibidi) 16 h before the experiment and grown to ~80% confluency. CPP-oligo nano complex was added to cells to

0.4 μM final concentration. At each time point, the images were collected on a confocal microscope (FV1000, Olympus) with a UPLFLN 40 $\times$ oil objective, for both Cy3 (555 nm–625 nm bandpass filter) and Cy5 FRET (650 nm long-pass filter) emission using a 543 nm HeNe laser at 10–20% power.

Apoptosis was introduced with staurosporine (STS). Three hours after probe loading, STS was added to cells to a final concentration of 2 μM . Fluorescence images were collected with above-mentioned confocal microscope settings.

The pixel intensity of each image was quantified in ImageJ. Each cell of interest was manually contoured using freehand selection tool and added to an ROI manager. Typically 80–130 cells were included from each image. The mean value was read from the measure function in the software.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

(a) Schematic of protease-activated oligonucleotide probe. Hairpin structure incorporates caspase-3 peptide substrate, Cy3-Cy5 FRET pair, and biotin affinity tag. Protease activity promotes capture (yellow) strand release and mRNA (purple strand) binding. (b) Reaction scheme and molecular structure. The capture strand (yellow) and blocking strand (green) were conjugated through CuAAC click reaction in good yield. Hairpin is stabilized by the neutral PNA backbone and terminal GC pair.

Figure 2.

(a) HPLC trace after PNA-oligonucleotide conjugation reaction. The product eluted at around 29 min with absorbance shown for 254, 552, and 643 nm. (b) ESI mass spectrum of final product.

Figure 3.

(a) FRET measurement, pre- and post-enzyme-mediated proteolysis. Caged form gave high FRET signal not influenced by poly-A RNA. FRET signal decreased after enzyme activation. (b) T_m measurement monitored through FRET change. T_m decreased from 69 to 34 °C after proteolysis. (c) Denaturing gel. Probe activated after incubation with caspase-3 for 4.5 h.

Figure 4. Confocal fluorescence micrographs 8 h after staurosporine treatment. (a) Confocal images, Cy3 and Cy5 channels. A significant decrease in FRET indicated enzyme activation. Scale bars represent 20 μm . (b) Average FRET and fluorescence intensity of Cy3 and Cy5 measured in cells.