Bioactivatable reductive cleavage of azobenzene for controlling functional dumbbell oligodeoxynucleotides

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ABSTRACT
Application of stimuli-responsive bioactive molecules is an attractive strategy due to use for target special tissues and cells. Here, we reported synthesis of an azo-linker, 2,2′-dimethoxyl-4,4′-dihydroxyazobenzene (mAzo), which was more effectively recognized and cleaved by reducing glutathione (GSH) via comparing with 4,4′-dihydroxyazobenzene (Azo). In addition, mAzo is further exploited to engineer dumbbell asODNs, which could result in the release of asODNs and thus modulate their hybridization to target nucleic acids. The present study is the first example to disclose efficient reductive cleavage of azobenzene by GSH to generate aromatic amine. This would provide a valuable strategy for tunable cell-specific release of ODNs and modulation of known disease-causing gene expression in cancer cells.

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1. Introduction
Artificial control of biofunction is one of the currently important and attractive themes for post-genome era, because of its potential application to gene therapy, biotechnology, and nanotechnology. In numerous systems, this is achieved through application of external stimuli such as pH [1–3], temperature [4,5], electric field [6,7] and light [8,9]. Especially, Biological stimuli, endogenously produced in specific cell types, present an attractive alternative to meet this goal due to their high selectivity, substrate specificity and mild operating conditions. Therefore, potential manipulation on the gene in different cells or tissues through regulation of endogenous biological substances will arrest more and more attentions.

In this strategy, an azobenzene linkage is introduced, as an artificial active site, which can be used as a powerful tool for studying living systems. Currently, numerous azobenzene derivatives have used as a useful core in several photochemical studies, and those systems. Currently, numerous azobenzene derivatives have usually served as a useful core in several photochemical studies, and those switches are used to control conformational changes in oligonucleotides [10–13], as well as functional outcomes [14–16]. However, certain sides effects on tissues and cells of using UV light in vivo have limited the application of such light-responsive molecules in biological systems. Therefore, numerous efforts are devoted to prepare azobenzene compounds, that operated with longer wavelengths of light or resistant to the reducing intracellular environment, enabling photocontrol of molecular processes in intracellular environments [17–20].

Additionally, there were studies to show that azo bond could be scissored by azoreductase/NADPH, produced by the microbial flora present in the colon of the human intestine [21,22], or present in the cytoplasm of hepatic cancer cells [23,24], which is being used in the treatment of colon diseases or liver cancers. Additionally, sodium di-thionite as a reducing reagent for azo bond, have been used to create a useful molecular device in fluorescent probes [25,26], therapeutic reagent [27,28] and nanocarrier fields [29,30]. Reductive cleavage of azo bond by hydrazine can be applied in polymer assemblies spheres [31]. However, few studies have reported the use of biological stimuli such as GSH to release active molecules in oligodeoxynucleotides, broadening applicability of these functional residues in vivo applications.

We previously reported an effective azobenzene photoswitch, 4,4′-dihydroxyazobenzene (Azo), which can stabilize the hairpin structure [12,13]. Hence, to preserve the conformational stability of ODNs associated with this unit, we utilize Azo as a nucleus to develop azobenzene linkers. A comparative investigation of GSH-induced reduction of Azo and its derivative, mAzo, was performed by UV–Vis spectra, fluorescence spectroscopy, and HPLC analysis. Furthermore, the azobenzene linkers were inserted between 18-mer asODNs and different short protective strands, forming a series of activatable dumbbell oligodeoxynucleotides. The reductive cleavage of azobenzene by GSH could result in the release of ODNs and thus modulate the
hybridization of asODNs to target DNA molecules. These findings would be valuable for further developing GSH-responsive DNA-based molecular devices as treatments for cancer and other diseases in human clinical trials.

2. Results and discussion

2.1. Design and synthesis of azobenzene derivatives and azobenzene linked dumbbell asODNs

In recent photochemical studies of azobenzene conjugates, much effort focuses on how to resist to reduction by GSH [17–20], resulting in more adaptation to intracellular environment. On the other hand, these aromatic azo-linkers are engineered to be effectively cleaved by biological stimuli, releasing the loaded functional molecules selectively [25,27,30,32–35], which can be used for site specific drug delivery [21,22,36,37] and polymer self-assembly [38–40]. Earlier reports showed that incorporating the electron-rich substituents of aromatic azobenzene will increase their susceptibility to cleavage by azoreductase enzymes [24]. When the electron-donating oxygen (O) or nitrogen (N) groups in the para and ortho positions were incorporated into the aromatic azobenzene ring, the cleavage level of azobenzene bond was improved.

In this work, Azox has served as a useful core in which two terminal hydroxydimethyl groups as reactive group were able to covalently attach to oligodeoxynucleotides. In order to identify the sensitivity of azobenzene with GSH, di-electron-donating oxygen (−OCH3) groups in the ortho positions of azobenzene were incorporated to control the electronegativity of azobenzene derivatives and enhance their cleavage rates by reduction agents.

To test effective cleavage of mAzo in system with oligodeoxynucleotides, the azobenzene unit is inserted between 18-mer asODNs and 4, 5 and 6 short protective strands, respectively. Similar to our previous report [13], a series of azobenzene linked dumbbell asODNs were formed, and the antisense ODN strands were temporally sequenced. In the presence of GSH, azo bonds were cleaved to respective aromatic amine, and two terminal protective strands of ODN hairpins were easily detachable, resulting in release of the asODNs, allowing asODN fragments to hybridize their complementary target DNA (Scheme 1).

mAzo was synthesized from methyl 4-amino-3-methoxybenzoate, with pyridine as a ligand and CuBr as the catalyst following published protocols [41] (Fig. S1, ESI†). As control, synthesis of Azo referred to our previous report [13]. To incorporate the azo-linker in dumbbell asODNs, one alcohol group of two aromatic rings was protected by DMT group, and the other one followed by reaction with N,N,N′,N′-tetramisopropylphosphorodiamidite (Fig. S1, ESI†), which formed an analogue of DNA phosphoramidites to couple with general DNA monomers by solid-phase synthesis method. Thus, a series of azobenzene linked dumbbell asODNs were formed and shown in Fig. 1. The dumbbell asODNs were designated to be AZ18P-4, AZ18O-5 and AZ18O-6 for mAzo-linked 18-mer asODNs with 4–6 nt hairpin stems, and AZ18P-4, AZ18P-5 and AZ18P-6 for Azo-linked 18-mer asODNs with 4–6 nt hairpin stems, respectively.

2.2. Reductive cleavage of azobenzene derivatives by GSH

A comparative investigation of GSH-induced of Azo and its derivative, 2,2′-dimethyl-4,4′-dihydroxyethylazo-benzeno (mAzo), is performed by UV–Vis spectra, fluorescence spectroscopy and HPLC analysis. It is reported that the content of GSH in cancer cells is 10–14 mM [42,43]. To make it easier to observe the high cleavage efficiency for mAzo with methoxy groups at the ortho-position, the concentration of GSH was increased to cleave the azo compounds in vitro study. mAzo and Azo were incubated with 25 mM GSH in PBS buffer (0.1 M, pH 7.0) containing 2 µL DMSO at 37 °C for different times (0 h, 3 h, 6 h, 12 h, 18 h and 24 h). The results showed that UV absorbance of mAzo at 370 nm (λmax for mAzo) obviously decreased upon incubation with GSH for 3 h, and reached a plateau within 24 h, accompanied by color change of the reaction solution from golden to white (Fig. S2, ESI†). This indicated that the mAzo-linker was effectively reduced by GSH and the azo bond chromophore was removed from the molecular structure (Fig. 2A). In contrast, UV absorbance of Azo at around 340 nm (λmax for Azo) remained almost constant upon incubation with GSH for 24 h under the same conditions (Fig. 2B). In addition, the reaction processes of reductive cleavage for mAzo and Azo were monitoring by fluorescence emission spectra. Similarly, fluorescence intensity of reaction mixture of mAzo displayed a remarkable increase at 420 nm (λem for mAzo) when incubated with GSH for 24 h, where as fluorescence intensity at 440 nm (λem for Azo) of reaction system for Azo still kept at a constant value (Fig. 2C and 2D). Moreover, a characteristic band of N–H wagging vibration at 900–650 cm−1 for cleavage product was increased using FT-IR spectra when compared with the raw mAzo (Fig. S3, ESI†), which further confirms mAzo was reductively cleaved to aromatic amine.

The reductive cleavage of mAzo and Azo in response to GSH was further assessed by HPLC analysis. As shown in Fig. 3A, the peaks at 8.7 min and 7.9 min are identified as trans form and cis form of mAzo, respectively. Upon incubation of mAzo with GSH for 3 h, the new main peak appeared at 6.3 min and increasing incubation time caused a concomitant increase in the amount of main product, which followed a similar trend to that observed by UV and fluorescence analysis. The ESI-
upon incubation with GSH. The absorbance changes for mAzo and fluorescence changes for incubated at 37°C for 0 h, 3 h, 6 h, 12 h, 18 h and 24 h. MS (Fig. S4, ESI†) and GC–MS (Fig. S5, ESI†) analysis for the main Azo and Fig. 3.

buffer (0.1 M, pH 7.0) containing 2 μL DMSO.

cubated with GSH (25 mM) at 37°C for 0 h, 3 h, 6 h, 12 h, 18 h and 24 h in PBS

Fig. 2. UV–Vis absorbance spectra and fluorescence spectra of mAzo and Azo upon incubation with GSH. The absorbance changes for mAzo (A) and Azo (B) and fluorescence changes for mAzo (C) and Azo (D) were recorded when incubated with GSH (25 mM) at 37°C for 0 h, 3 h, 6 h, 12 h, 18 h and 24 h in PBS buffer (0.1 M, pH 7.0) containing 2 μL DMSO.

Fig. 3. HPLC analysis of the GSH-mediated cleavage reactions for mAzo (A) and Azo (B). HPLC results were recorded when GSH (25 mM) was added and incubated at 37°C for 0 h, 3 h, 6 h, 12 h, 18 h and 24 h.

MS (Fig. S4, ESI†) and GC–MS (Fig. S5, ESI†) analysis for the main cleaved product showed 4-amino-3-methoxyphenylmethanol was produced by azo bond cleavage. Differently, no new peak was observed for Azo incubated with GSH for 24 h (Fig. 3B).

To probe the cleavage level of azobenzene compounds in mimic cancer cell conditions, the degradation studies of mAzo and Azo were carried out in the presence of 10 mM reduced glutathione. We simultaneously used UV–Vis spectra and HPLC to monitor the azo bond cleavage in solution upon incubation of mAzo or Azo with GSH. Result revealed that incubation of mAzo with GSH has similar variation trend in the presence of 10 mM GSH (Fig. S6A and S6C, ESI†), although low concentration of GSH (10 mM) reacted with mAzo was less completely than high concentration of GSH (25 mM). By contrast, we could not detect the cleavage of Azo under the same conditions (Fig. S6B and S6D, ESI†). The observed results clearly showed that GSH indeed triggered the reductive cleavage for mAzo but not for Azo under the same conditions, which correlates with incorporation of electron-donating groups (–OCH3) in the ortho position.

Fig. 4. Proposed the reaction mechanism of mAzo with GSH.

Additionally, effect of the isomers of mAzo on GSH-induced reducing cleavage was assessed. The parallel mAzo solutions were added with GSH, irradiated respectively with UV (365 nm, 7 mW/cm²) and visible light (>400 nm, 11 W) for 5 min at room temperature, and then incubated under same conditions for various times, respectively. The HPLC data showed that mAzo mostly took a cis configuration upon UV irradiation where as it exists dominantly in the cis form with visible light irradiation at room temperature. This finding was explained by the slight difference between these two energy states in water according to the results of energy calculations of trans-mAzo and cis-mAzo in water and methanol (Figs. S9 and S10, ESI†). Furthermore, for two reaction systems of mAzo upon UV irradiation and visible light irradiation, the spectral changes displayed that UV absorbance at 370 nm gradually decreased after incubating with GSH until it reached a constant value after 24 h incubation (Fig. S11, ESI†), indicating both isomers of the mAzo were reduced by GSH. HPLC analysis displayed their reduction by GSH, which produced the amine product (Fig. S12, ESI†). What’s more, GSH-induced reaction with cis-mAzo has relatively higher reaction rate than trans-mAzo by quantitative analysis of cleaved products of mAzo after UV irradiation and visible light irradiation (Fig. S13, ESI†).

2.3. Mechanistic study

Prompted by the observation that proteinogenic thiols reduce azobenzene [44], we incubated mAzo with cysteine containing thiol as a positive control. Results showed that a reduction in UV absorbance was reduced (Fig. S7, ESI†), indicating that mAzo can be reduced by cysteine. HPLC analysis simultaneously showed that a new peak appeared at 6.3 min (Fig. S8, ESI†), corresponding to 4-amino-3-methoxyphenylmethanol. Comparing with cysteine, alanine without thiol did not induce change in UV absorbance of mAzo. These results collectively showed that the azo bond of mAzo was digested by thiol, which agreed with the mechanism of reduction by thiol for diazenedicarboxylic acid derivatives, presented initially by Kosower [45]. This process involved that the thiol attacked one azo nitrogen atom, and the second azo nitrogen atom became protonated to give a sulfonyl hydrazine derivative. However, mAzo was more electron rich in ortho substituted position, and thus protonation of the azo group occurred by prior, or concomitant, giving six-membered intermediate, due to the formation of H-bonding to the methoxyl group in the planar conformation (Fig. 4). In the present case, thiol attack is facilitated by increasing the electrophilicity of the diazo system. This leads to an unstable sulfonyl hydrazine intermediate, which is further reattached by a proton with the assistance of GS−, producing an unstable hydrazone intermediates. It is easily reduced cleaving the N−N bond into its respective amines with addition of H2O or GSH. Combined with the GSH and Cys results, we observed that the degree of reaction of mAzo with GSH was more completely than Cys, which would be attributed to the relatively low pH value of GSH reaction system, due to GSH has two carboxyl groups.

Moreover, effect of the isomers of mAzo on GSH-induced reducing cleavage was assessed. The parallel mAzo solutions were added with GSH, irradiated respectively with UV (365 nm, 7 mW/cm²) and visible light (>400 nm, 11 W) for 5 min at room temperature, and then incubated under same conditions for various times, respectively. The HPLC data showed that mAzo mostly took a cis configuration upon UV irradiation where as it exists dominantly in the cis form with visible light irradiation at room temperature. This finding was explained by the slight difference between these two energy states in water according to the results of energy calculations of trans-mAzo and cis-mAzo in water and methanol (Figs. S9 and S10, ESI†). Furthermore, for two reaction systems of mAzo upon UV irradiation and visible light irradiation, the spectral changes displayed that UV absorbance at 370 nm gradually decreased after incubating with GSH until it reached a constant value after 24 h incubation (Fig. S11, ESI†), indicating both isomers of the mAzo were reduced by GSH. HPLC analysis displayed their reduction by GSH, which produced the amine product (Fig. S12, ESI†). What’s more, GSH-induced reaction with cis-mAzo has relatively higher reaction rate than trans-mAzo by quantitative analysis of cleaved products of mAzo after UV irradiation and visible light irradiation (Fig. S13, ESI†).
2.4. GSH-induced azobenzene cleavage for application in dumbbell asODNs

AsODNs have been proven effective in gene silencing in many experimental systems and are being developed as treatments for cancer and other diseases in human clinical trials [46]. For the regulation of gene expression, an antisense strategy based on the hybridization of target mRNA with antisense DNA has been widely adopted. As expected, based on our previous work with azobenzene linked dumbbell asODNs [13], the dumbbell asODNs effectively controlled the DNA/RNA or DNA/DNA hybridization to regulate target RNA digestion through azobenzene photoisomerization. Since GSH is more widely existed in cancer cells than normal cells [5,47,48], we will seek to develop GSH-induced release of asODNs for modulating the hybridization of asODNs to target DNA or RNA molecules.

To confirm that reductive cleavage of azobenzene and release of asODNs are selectively triggered by GSH, the mAzo conjugates and Azo conjugates were incubated with GSH at 37 °C for 4 h, 8 h and 24 h while monitoring release of asODNs by denaturing polyacrylamide gel electrophoresis (PAGE). Fig. 5 showed results of reducing cleavage of these dumbbell asODNs in absence or presence of GSH. Lanes 1, 3, 5 and 7 were each panel showed the mobility of the dumbbell asODNs without and with GSH, respectively. Obviously, cleavage of mAzo linked dumbbell asODNs (AZ18O-4, AZ18O-5 and AZ18O-6) occurred upon incubation with GSH for 4 h and the major bands were the cleavage products of one mAzo-linker. With increasing incubation time, two mAzo-linkers were gradually cleaved and caused a significant increase of 18-mer asODNs after 24 h incubation (Fig. 5A). However, for Azo linked dumbbell asODNs (AZ18P-4, AZ18P-5 and AZ18P-6), there was no obvious increase of cleaved bands within 8 h under the same GSH assay conditions and even no product bands of 18-mer asODNs after 24 h (Fig. 5B). This clearly shows that GSH reduction of the mAzo-linker is the “trigger” responsible for asODN release. By quantifying the percentage of cleaved dumbbell asODNs after incubating for 24 h, 87.8%, 88.0% and 88.9% of mAzo linkers were cleaved for AZ18O-4, AZ18O-5 and AZ18O-6 by GSH, while only 24.4%, 23.8% and 20.3% dumbbell asODNs were reductively cleaved for AZ18P-4, AZ18P-5 and AZ18P-6. This indicated that the cleavage efficiencies were almost independent of numbers of binding arms for the dumbbell asODNs.

To further confirm GSH-induced binding target DNA with azobenzene linked dumbbell asODNs, based on many biological activities of asODNs involving the hybridization, GSH-induced release of asODNs for modulating the hybridization between asODN and target DNA or RNA was investigated using native PAGE. Different from existed form of stable isomers of only mAzo, mAzo in dumbbell asODNs displayed the main conformation in trans state without irradiation, according to the photoisomerization of mAzo linked dumbbell asODNs with UV and visible light irradiation (Fig. S14, ESI). The results of these dumbbell asODNs binding to target DNA (MB-7) in absence or presence of GSH at 37 °C were shown in Fig. 6. With this system, these asODNs with dumbbell structures should lead to binding competition to asODN fragments between two binding arms and their target DNA. As the number of binding arms increased for the dumbbell ODNs (AZ18O-4, AZ18O-5 and AZ18O-6), the binding ability between dumbbell asODN and target DNA (MB-7) was weakened (Fig. 6A, Lanes 2, 4 and 6). Indeed, Fig. 6A showed AZ18O-6 (2.0%) with two longer binding arms produced much lower “background” binding than AZ18O-4 with two shorter binding arms (54.3%).

In addition, as shown in mAzo-linker’s reduction (Fig. 3A) and the associated ODN release profile (Fig. 5A), the total amount of binding products of AZ18O-4, AZ18O-5 and AZ18O-6 with MB-7 showed slight differences upon incubation of the dumbbell ODNs with GSH (Fig. 6A, Lanes 3, 5 and 7). However, incubation of the dumbbell ODNs with GSH for 24 h can distinctly distinguish the difference in binding of the dumbbell ODNs to MB-7. For example, the incubation with GSH caused an increase in binding products of AZ18O-4, AZ18O-5 and AZ18O-6 from 54.3%, 14.2% and 2% to 80.5%, 67.6%, and 57.6%, which were about 1.5, 4.8 and 28.8-fold increase, respectively (Fig. 6A). Specifically, of the different dumbbell ODNs studied, AZ18O-6 with 6 nt nucleobases in binding arms was by far most efficient for binding to target DNA. This indicated that the GSH-induced dumbbell ODNs for binding to target gene depended on not only azobenzene linker but also the length of protective strand of the dumbbell ODNs. Compared with regulation of the target DNA using the dumbbell asODNs upon light irradiation [13], GSH-mediated activation of the dumbbell asODNs showed much higher efficiency. For Azo system, with the numbers of binding arms increased, binding ability of dumbbell asODNs and target MB-7 followed a same trend as mAzo system in the absence of GSH (Fig. 6B, Lanes 2, 4 and 6). On the other hand, no much stronger hybridization bands were observed for AZ18P-4, AZ18P-5 and AZ18P-6 with GSH reduction, which indicates that all of the Azo modified asODNs were less sensitive to GSH.

3. Conclusions

In summary, 2,2′-dimethoxyl-4,4′-dihydroxymethyl (mAzo) described here can be more effectively cleaved by reductive glutathione. Furthermore, an “intelligent” azobenzene-conjugate system with oligodeoxynucleotides was engineered to yield asODNs to bind target DNA with respond to GSH. Taken together, the current research on the ortho-methoxyl substituted azobenzene derivatives provided a novel strategy on GSH-induced molecular responses, and its attachment to a wide variety of targets can be extended versatility of azobenzene for in vivo use. Interestingly, intracellular GSH concentration is much higher in cancer cells than normal cells. The reductive cleavage of azobenzene will be important for controlling cancer gene and developing prodrug.

![Fig. 5. 20% denaturing PAGE of the cleavages of mAzo (A) and Azo (B) linked dumbbell asODNs with incubating 25 mM GSH at 37 °C for 4 h, 8 h and 24 h. “−” and “+” correspond to the dumbbell asODN without and with GSH, respectively.](image-url)

![Fig. 6. 20% Native PAGE of the binding of mAzo (A) and Azo (B) linked dumbbell asODNs (2μM) with MB-7 (2μM) in 1× PBS buffer at 37 °C after 24 h incubation. Lane 1: MB-7, “−” and “+” correspond to the dumbbell asODN and MB-7 without and with GSH, respectively.](image-url)
4. Experimental

4.1. Chemicals and techniques

All the reagents for organic synthesis were analytically pure and solvents for synthesis and purification were distilled over CaH₂. All oligonucleotides were custom-synthesized on ABI 394 DNA/RNA synthesizer (Applied Biosystems). Deprotected oligonucleotides were purified with an Agilent 1260 system using Agilent reverse phase C18 column (5 μm bead, 9.6 mm × 150 mm). The UV–Vis spectra were measured on a Shimadzu UV-1800 model spectrophotometer. All fluorescence spectra were recorded in JOBIN YVON Fluoromax-4. Analytical PAGE was carried out on BioRad ChemiDoc™ MP Imaging System. UV irradiation in vitro experiments were carried out using UV-LED lamp (365 nm, 7 mW/cm²). Visible irradiation was performed using general energy-efficient lamp (> 400 nm, 11 W). ¹H NMR and ¹³C NMR spectra were recorded using JNM-ECZ400S (400 MHz for ¹H, 100 MHz for ¹³C). Mass spectra were obtained using GC–MS and ESI-MS.

4.2. Synthesis

4.2.1. Synthesis of compound 1

CuBr (14.3 mg, 0.1 mmol), pyridine (23.7 mg, 0.3 mmol), and methyl 4-amino-3-methoxybenzoate (362 mg, 2.0 mmol) were mixed in 14.2.2. Synthesis of compound 2

Of dimethyl 4,4′-dimethoxytrityl chloride (DMT-Cl, 169 mg, 0.5 mmol) was added, and the solution stirred for an additional 2 h at 60 °C. After the reaction was completed, all solvents were evaporated under reduced pressure. The crude product was purified by column chromatography on silica gel using petroleum ether and ethyl acetate (V/V = 8:1) as eluents, to afford 89.5 mg (in yield 25%) of dimethyl 4,4′-diazene-1,2-diyl)-bis(3-methoxybenzoate) as a red solid. ¹H NMR (CDCl₃, 400 MHz) δ 7.76 (d, J = 1.4 Hz, 1H), 7.68 (dd, J = 1.2 Hz, 8.0 Hz, 1H), 7.62 (d, J = 8.4 Hz, 1H), 4.08 (s, 3H), 3.95 (s, 3H). ¹³C NMR (CDCl₃, 100 MHz) δ 166.51, 156.66, 145.38, 133.57, 122.27, 117.55, 113.64, 56.55, 52.61.

4.2.2. Synthesis of compound 2

300 mg (0.84 mmol) of intermediate (dimethyl 4,4′-diazene-1,2-diyl)-bis(3-methoxybenzoate)) was dissolved in 20 mL dry THF, then 95.63 mg (2.52 mmol) of LiAlH₄ was slowly added into solution while vigorously stirred at 60 °C for 24 h. After cooling down to room temperature and concentrating under vacuum, the crude product was purified by column chromatography on silica gel using petroleum ether and ethyl acetate as eluents. The synthesized compound was then purified by column chromatography on silica gel using petroleum ether/ethyl acetate (1:9) and concentrated under reduced pressure to obtain a yellow solid. The crude product was purified by silica gel chromatography with petroleum ether/ethyl acetate as eluent. The purified product was then concentrated at 40 °C and a gradient of acetonitrile (B): 0–40% in 40 min, 40–100% in 45 min, 100% in 50 min, 100%–0% in 55 min, 10% in 60 min. The objective preparative eluent was collected after the analysis running on HPLC was monitored for UV absorbance at 260 nm. All the obtained oligonucleotides were characterized by ESI-MS under negative mode (1 μL sample (100 μM) in H₂O/CH₃CN (1:1) and 1% TEA). The amounts of DNA were determined by UV absorbance at 260 nm using NanoDrop (Thermo scientific).

4.2.3. Synthesis of compound 3

mAzo (300 mg, 1.0 mmol) was dissolved in anhydrous THF (5 mL), and Triethylamine (TEA, 100 μL) was added to the solution and then 4,4′-dimethoxytrityl chloride (DMT-Cl, 169 mg, 0.5 mmol) was added to the mixture, which was stirred for 3 h at 60 °C. After monitoring the reaction progress by thin-layer chromatography (TLC), DMT-Cl (119 mg, 0.35 mmol) was added, and the solution stirred for an additional 2 h at 60 °C. After the reaction was completed, all solvents were evaporated under reduced pressure. The crude product was purified by silica gel column chromatography (petroleum ether/ethyl acetate/Triethylamine = 25:10:1) to obtain compound 3 as a red solid (242 mg) in yield 80%. ¹H NMR (CDCl₃, 400 MHz) δ 7.64 (d, J = 4 Hz, 1H), 7.62 (d, J = 4 Hz, 1H), 7.52 (d, J = 8 Hz, 2H), 7.42 (d, J = 4 Hz, 2H), 7.40 (d, J = 2 Hz, 2H), 7.31 (d, J = 7.6 Hz, 2H), 7.22 (d, J = 7.2 Hz, 1H), 7.11 (d, J = 5.6 Hz, 2H), 7.01 (d, J = 8.4 Hz, 1H), 6.95 (d, J = 8.4 Hz, 1H), 6.87 (s, 2H), 6.84 (s, 2H), 4.75 (s, 2H), 4.24 (s, 2H), 4.02 (d, J = 3.2, 6H), 3.80 (s, 6H), 13C NMR (CDCl₃, 100 MHz) δ 158.59, 157.05, 156.91, 145.01, 144.32, 142.21, 141.86, 136.20, 130.14, 128.22, 128.03, 126.96, 119.12, 118.94, 117.61, 117.37, 113.27, 110.79, 110.69, 86.71, 65.53, 65.09, 56.41, 55.34.

4.2.4. Synthesis of azobenzene linked dumbbell asODNs

The oligonucleotides were treated with concentrated ammonium hydroxide for 12 h at 50 °C to cleave them from solid supports and deprotect the phosphates and nucleobases. The solid supports were then filtered out and the filtrates were concentrated to dryness. Oligonucleotide purification was performed by a RP-HPLC with Agilent reverse phase C18 column (5 μm, 9.4 mm × 250 mm) using 0.05 M triethylammonium acetate (TEAA, phase A) and acetonitrile (phase B) as eluents. The samples eluted at 1.0 mL/min with running temperature at 40 °C and a gradient of acetonitrile (B): 0–40% in 40 min, 40–100% in 45 min, 100% in 50 min, 100–10% in 55 min, 10% in 60 min. The objective preparative eluent was collected after the analysis running on HPLC was monitored for UV absorbance at 260 nm. All the obtained oligonucleotides were characterized by ESI-MS under negative mode (1 μL sample (100 μM) in H₂O/CH₃CN (1:1) and 1% TEA). The amounts of DNA were determined by UV absorbance at 260 nm using NanoDrop (Thermo scientific).

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4.2.6. UV–Vis spectra and HPLC characterization

A stock solution (3.3 mM) of mAzo and Azo in DMSO was prepared, then added 30 μL GSH (25 mM), 2 μL PBS (0.1 M, pH 7.0), and 4 μL MgCl₂ (5 mM) to 600 μL bottle. After completed, the reaction was diluted with ultrapure water to obtain the desired concentration, and the change of absorbance was monitored using an UV1800 spectrophotometer. At the same time, the same samples were analyzed using a C18 (5 μm, 4.6 × 250 mm) column connected to an Agilent HPLC system equipped with a UV dual absorbance detector. A mixture of water and acetonitrile was used as a mobile phase to separate product and crude material on the C18 column using a H₂O:ACN gradient of 30:90 in 10 min, 10:90 in 11–14 min, and 90:10 in 15–17 min at a flow rate of 1 mL/min. Absorbance of eluting reductive cleavage product molecules was measured at 287 nm.

4.2.7. Polyacrylamide gel electrophoresis (PAGE) characterization

GSH (25 mM) were added to equimolar solutions of azobenzene modified dumbbell ODNs (2 μM) dissolving in PBS buffer (0.1 M, pH = 7.0) and MgCl₂ (5 mM), and then respectively incubated for 4 h,
8h, 24h at 37°C while shaking at 210rpm. Before the samples were loaded into the gel, appropriate 2 × glycerol gel loading buffer containing bromophenol blue was added. All appropriate solutions were loaded into 20% denaturing polyacrylamide gels containing 7 M urea. The gels were then electrophoresed at 150 V for 1.5 h at room temperature, using 1 × TBE buffer. Gels were imaged with a BioRad ChemiDoc™ MP Imaging System.

4.2.8. Binding studies

The solutions of the fluorescein labelled MB-7 (2 μM) with azobenzene modified ODNs (2 μM) and GSH (25 mM) in 4 μL 1 × PBS buffer was used for binding assays. The mixture solution was incubated at 37°C for 24 h and was then concentrated. Before the samples were loaded into the gel, 1 μL 6 × sucrose gel loading buffer containing bromophenol blue was added. All 6 μL solutions were loaded into 20% native polyacrylamide gels. The gels were then electrophoresed at 150 V for 1.5 h at room temperature, using 1 × TBE buffer. Gels were imaged with a BioRad ChemiDoc™ MP Imaging System.

Supporting information

The supporting information is available online at http://chem.scichina.com and http://link.springer.com/journal/11426. The supporting information are published as submitted, without typesetting or editing. The responsibility for scientific accuracy and content remains entirely with the authors.

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Declaration of Competing Interest

The authors declare that they have no conflict of interest.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioclr.2019.103106.

References


