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New quinoline-based caging groups synthesized for photo-regulation of aptamer activity

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ABSTRACT

A series of quinoline-based photo-removable protecting groups for photo-regulation of thrombin aptamer (HD1) activity were synthesized with improved caging and uncaging efficiency. Among them, 8-bromo-2-diazomethyl-7-hydroxyquinolinyl (BHQ-diazo, **1**) chromophore was found to cage the HD1 with highest caging and restoration efficiency. Moreover, on the basis of the RP-HPLC and SPR analysis, BHQ was demonstrated to regulate HD1s specific affinity to target molecule with 3-fold photolysis sensitivity and about 40% percent higher uncaging efficiency than Bhc (6-bromo-7-hydroxycoumarin-4-ylmethyl) group. It was proposed that the development and use of quinoline derivative may provide a general strategy to photo-regulate oligonucleotide's activity with improved caging and uncaging efficiencies by the convenient non-site-specific caging method.

1. Introduction

Photochemical approaches for controlling bio-molecule functions have become increasingly important in elucidating complex biological processes [1–5]. Generally, molecules were encapsulated in an inactive form by being caged and subsequently, restored to display biological activity with irradiation. Recently, many studies on "up- and down-photoregulating" DNA and RNA functions, including DNA hybridization [6,7], polymerase [8–10], RNase H activity [11,12], RNA interference [13–17], siRNA [16,18] with exact spatiotemporal resolution, have already been carried out. In 1999, Monroe et al. [19] provided the first example of targeting gene expression with light in a biological system by none site-specific (multiple) caging strategy using DMNPE. Ando et al. [20,21] applied the similar strategy to control gene expression in zebrafish embryos by Bhc protecting group and 23% activity was restored after 20 s irradiation with high intensity UV light. Recently, Monroe [6,15] further accomplished photoregulation of DNA hybridization and RNA interference in vitro and in vivo with a better uncaging efficiency. Simultaneously, site-specific caging strategy was developed and performed by Heckel [2,7,16,22,23], Dmochowski [1,8,11,24–28], Deiters [9,29–33], Chen [34] groups by using a single photoactive group. Through complicated and time-consuming caging processes, higher caging and uncaging efficiencies could be realized. Unfortunately, most single photoactive groups proved to be too stable, requiring longer irradiation time (at least 5 min) for releasing the compounds, which should be the primary obstacle to this method [35].

Photo-regulation of aptamer (HD1) activity was firstly processed by Heckel and Mayer using site-specific method [23]. HD1, derived from an evolution process called SELEX (Systematic Evolution of Ligands by Exponential enrichment), shows a high affinity and specific binding to target molecules and inhibits their biological functions. These features make HD1 a promising probe for molecular recognition as well as diagnostic and therapeutic applications [36,37]. In the previous work, thrombin HD1, a 15mer ssDNA molecule (5′-GGTTGGTGTGGTTGG-3′), was caged by one-photon compound o-nitrophenylpropyl (NPP) or o-nitrophenylethyl (NPE) by solid-phase approach [23]. This site-specific caging method gains control over the spatially and temporally high resolution availability of the HD1s function. However, due to the drawback described above, the excessively longer irradiation time (25 min) was required for activity restoration. Besides, this strategy is limited by complicated processes and unavailable for potential two-photon uncaging.

Recently, our group developed a method to photo-regulate thrombin HD1 activity by Bhc caging strategy. Through co-incubation of Bhc-diazo with HD1 within 2 h, the specific activity of HD1 was successfully blocked and the effect was partially restored with subsequent 1 min illumination [38]. However, insufficient uncaging efficiency (less than 60%) was observed presumably due to various negative properties of Bhc group and therefore, became

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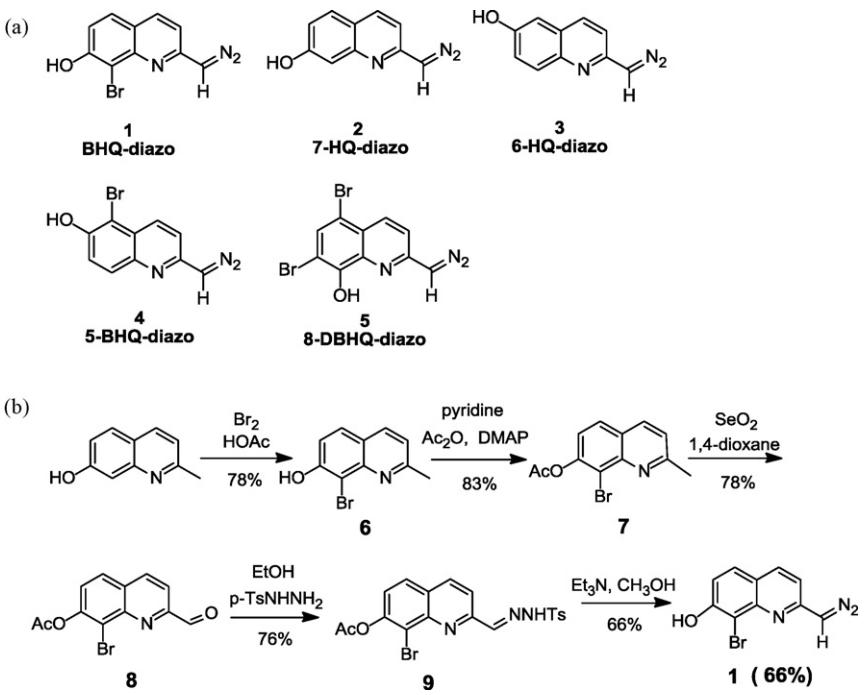

Fig. 1. The structures of compounds **1**–**5** (a) and the synthesis procedures of compound **1** (b).

the primary obstacle to biological studies. As Dmochowski group reported, this incomplete uncaging was caused by the difficulty of removing most of the caging group with non-toxic exposures to UV light [35]. To solve these problems, we prefer to develop some modified compounds with less reactive equivalency and better photolysis sensitivity for improvements of blockade and restoration efficiency [15,38]. Among previously reported groups for none site-specific caging strategy, 8-bromo-7-hydroxyquinolinyl chromophore (BHQ), which was firstly synthesized by Dore and co-workers for photo-releasing acetate, phosphates, and diols-functional groups [39–41], was found to have a larger two-photon across section, a higher photolysis sensitivity, and an improved water solubility.

In the present study, we synthesize the diazomethane form of BHQ (BHQ-diazo) to cage with thrombin HD1 and further photoregulate its activity. Moreover, through changing the position of bromine and hydroxyl groups, a series of quinoline-based derivatives were developed for the purpose of selecting the higher photolysis sensitivity compounds for multiple caging with nucleic acid.

2. Experiments

2.1. Chemistry and instrumentation

All solvents used for reactions were purchased as anhydrous grade from Fluka and were used without further processing. Solvents for extractions, column chromatography, and TLC were commercial grade and distilled before use. TLC was performed on Merck TLC aluminum sheets (silica gel 60 F254). Spots were visualized with UV light or through staining with phosphomolybdic acid or $KMnO_4$. Flash column chromatography (FC) was performed using Fluka silica gel 60 for preparative column chromatography (40–63 mm), unless specifically noted otherwise. Proton NMR spectra were obtained on a Varian Unity 400 (400 MHz) spectrometer with use of $CDCl_3$ as solvent and tetramethylsilane as an internal standard. Carbon-^{13}NMR spectra were performed on a Varian Unity 400 (100 MHz) spectrometer with use of $CDCl_3$ as solvent. Carbon-13 chemical shifts are referenced to the centre of the $CDCl_3$ triplet (d 77.0 ppm).

2.2. Synthesis

2.2.1. The synthesis of BHQ-diazo (*1*)

On the basis of above proposal, we synthesized compound **1**–**5**. To provide an example of synthetic procedures, BHQ-diazo **(1)** was prepared from 7-Hydroxyquinaldine and described as follows (Fig. 1). Compounds **2**–**5** were synthesized by using similar synthetic procedures.

8-Bromo-2-methylquinolin-7-ol **(6)**: The solution of bromine (6 mmol) in the glacial acetic acid (20%) was added to the solution of the 7-hydroxyquinaldine (5.5 mmol) in the glacial acetic acid (15 mL) dropwise with vigorous stirring. The precipitate appeared during reaction. The mixture was stirred for 8 h, then mixture was diluted with $CHCl_3$ and washed with saturated $NaHCO_3$, water, and brine. The organic layer was dried with Na_2SO_4 and evaporated which was purified by silica gel with EtOAc/petroleum ether (1:4) to give product **6** (78% yield).

8-Bromo-2-methylquinolin-7-yl acetate **(7)**: The compound **6** (4 mmol) and DMAP (0.4 mmol) were dissolved in pyridine (20 mL) under argon atmosphere. Followed by the acetic anhydride (6.0 mmol) slowly added to the reaction mixture, the reaction was stirred for 4 h at room temperature. The mixture was diluted with $CHCl_3$, washed by 15% citric acid, water, and brine, and the organic layer was dried over Na_2SO_4. The solvent was removed by rotary evaporation, and the crude product was purified over silica gel using EtOAc/petroleum ether (1:2) to give product **7** (83% yield).

8-Bromo-2-formylquinolin-7-yl acetate **(8)**: The selenium dioxide (2.04 mmol) was dissolved in 1,4-dioxane (4 mL), and a solution of compound **7** (2.0 mmol) in 1,4-dioxane (2.5 mL) was added to the mixture at 60 °C under argon. The temperature was allowed to rise to 80 °C, and the mixture was stirred for 8 h. The precipitate was removed by filtration, and the filtrate was evaporated to give the crude product which was purified by flash column chromatography (EtOAc/petroleum ether; 1:4) to gave **8** (78% yield).

8-Bromo-2-((2-tosylhydrazono)methyl)quinolin-7-yl acetate **(9)**: The compound **8** (1 mmol) was dissolved in EtOH (6 mL). p-Toluenesulfonyl hydrazine (1.06 mmol) was added to the mixture. The reaction was stirred for 19 h at 40 °C. The precipitate was

collected by filtration, washed with EtOH, and dried under vacuum to yield **9** (76% yield).

8-Bromo-2-(diazomethyl)quinolin-7-ol (**1**): The compound **9** (3.0 mmol) was dissolved in MeOH (7 mL) at room temperature. Et_3N (6.0 mmol) was then added to the suspension. The reaction was stirred for 2 days. Then the precipitate was collected by filtration, washed with CH_3OH, and dried under vacuum to yield **1** (66% yield).

*2.2.2. The analytical data of compounds **1**–**5***

The ^{1}H NMR (400 MHz, DMSO, δ ppm) of **1**: 11.33 (s, 1H), 8.27 (s, 1H), 7.93 (d, J = 8.7, 1H), 7.68 (q, J = 9.2, 2H), 7.35 (d, J = 8.7, 1H); The ^{13}C NMR (100 MHz, DMSO, δppm) of **1**: 157.2, 133.6, 131.8, 129.8, 127.7, 126.3, 119.3, 115.9, 112.45, 96.8. HRMS calcd for $C_{10}H_6N_3O^{79}Br$: 262.9694; found: 262.9702.

The ^{1}H NMR (400 MHz, DMSO, δppm) of **2**: 10.74 (s, 1H), 8.24 (s, 1H), 8.01 (d, J = 2.0, 1H), 7.94 (d, J = 8.7, 1H), 7.70 (d, J = 9.2, 1H), 7.60 (d, J = 9.2, 1H), 7.19 (dd, J = 8.7, 2.0, 1H); The ^{13}C NMR (100 MHz, DMSO, δppm) of **2**: 159.6, 132.4, 132.0, 130.9, 127.2, 126.9, 117.3, 116.5, 111.4, 100.2. HRMS calcd for $C_{10}H_7N_3O$: 185.0589; found: 185.0586.

The ^{1}H NMR (400 MHz, DMSO, δppm) of **3**: 10.16 (s, 1H), 8.52 (d, J = 8.8, 1H), 8.23 (s, 1H), 7.75 (d, J = 9.3, 1H), 7.66 (d, J = 9.3, 1H), 7.33 (dt, J = 8.8, 2.5, 2H); The ^{13}C NMR (100 MHz, DMSO, δppm) of **3**: 156.5, 130.8, 127.6, 126.5, 125.5, 124.7, 119.7, 117.0, 115.4, 112.4. HRMS calcd for $C_{10}H_7N_3O$: 185.0589; found: 185.0596.

The ^{1}H NMR (400 MHz, DMSO, δppm) of **4**: 11.02 (s, 1H), 8.59 (d, J = 9.1, 1H), 8.31 (s, 1H), 7.90 (s, 2H), 7.51 (d, J = 9.1, 1H); The ^{13}C NMR (100 MHz, DMSO, δppm) of **4**: 153.8, 130.6, 127.9, 125.4, 125.0, 124.3, 119.4, 117.1, 116.3, 107.5. HRMS calcd for $C_{10}H_6N_3O^{79}Br$: 262.9694; found: 262.9701.

The ^{1}H NMR (400 MHz, DMSO, δ ppm) of **5**: 11.56 (s, 1H), 8.52 (s, 1H), 8.23 (s, 1H), 8.03 (d, J = 9.6, 1H), 7.90 (d, J = 9.6, 1H); The ^{13}C NMR (100 MHz, DMSO, δ ppm) of 5: 168.8, 134.4, 133.2, 128.0, 127.2, 124.8, 120.3, 119.7, 118.0. HRMS calcd for $C_{10}H_6N_3O_{79}Br_2$: 340.8799; found: 340.8797.

The NMR spectrums of compounds **1**–**5** were shown in e-component.

2.2.3. HD1 caging with compound

Thrombin HD1 (5′-GGTTGGTGTGGTTGG-3′) was synthesized as previously described [23]. A 5 μl volume of the **1**–**5** (25 μg/μl in DMSO) solution was mixed with 5 μl HD1 (1 μg/μl in 4:1 DMSO–aqueous) and the solution was incubated for 6–8 h at room temperature respectively. To remove excess caging compound, buffer and organic solvent from the reaction, the mixture was purified using Microcon YM-3 (3000 MW cutoff) centrifugal filters (Millipore, Bilerica, MA). The solution (10 μl) was diluted with DMSO (240 μl) and water to achieve a 1:1 DMSO–aqueous solution and spun at 12,000 × g for 100 min. Then the caged HD1 was re-suspended with aqueous solutions (4× 500 μl) and spun at 12,000 × g for another 200 min. All products were stored at 4 °C and protected from light.

2.2.4. Light-activation and spectrophotometric analysis of caged HD1

Absorption spectrophotometric analysis of caged species was used to estimate the degree of caging. Native (noncaged) and caged HD1 were dissolved in aqueous in separate cuvettes and scanned for absorbance from 240 to 500 nm using a spectrophotometer (U-2810; Hitachi). Light-activated HD1 was exposure to ultraviolet light by illumination with the 100-W mercury lamp of a microscope (Carl Zeiss). The intensity of the light is nearly 10% of the maximum intensity of mercury lamp and the does is approximately 100 mJ/cm^2. The selected spectrum of the light is 365 ± 5 nm and scans with similar methods as described above [20,42]. To determine the spectral changes following photoactivation, some of the light-activated HD1 products were further purified with the Microcon YM-3 filters following light exposure to remove the released compound, and then scanned as described. These samples are mentioned as purified light-activated HD1.

2.2.5. Gel electrophoresis of caged HD1

Three species of HD1 (250 ng) were run in a 12% polyacrylamide-urea denaturing gel in Tris-borate (TBE) buffer (100 mM tris-borate, 2 mM EDTA pH 8.5) at 80 V for 100 min. Gels were stained after electrophoresis with 1× Gel-red nucleic acid gel stain (Molecular Probes) in NaCl buffer (100 mM) for 8–10 min.

2.2.6. Determination of photolysis efficiency

RP-HPLC was used to evaluate the control, caged, and light-activated HD1. Each sample type was injected (1.25 mg, 25 mL) in HPLC-grade water into a C18 analytical column, ODS2 spherisorb 5 μm (Waters Corporation, Milford, MA). Separation was obtained using a gradient of 1% TEAA in HPLC water to 1% TEAA in 40% acetonitrile over 15 min.

2.2.7. Study the specific affinity of HD1 by SPR assay

SPR sensorgrams measurements were carried out at 25 °C using BIAcore 3000 (GE Healthcare). Before immobilization, PBS was used as the running buffer. The carboxyl groups on the chip surface were activated by injecting a freshly prepared solution of 50 mM NHS and 200 mM EDC in distilled water for 20 min. Then 0.2 M ethylene diamine in 50 mM sodium borate buffer (pH 8.5) was injected for 20 min to react with the activated chip surface. The remaining active groups on the chip surface were then deactivated by injecting 1 M ethanolamine hydrochloride solution in distilled water (pH 8.5) for 10 min. Subsequently, thrombin was diluted with PBS (PH 7.4) to 25 ng/μl and immobilized onto the CM5 sensor chip (GE Healthcare). In each measurement, control, caged or light-activated HD1 was passed through sensor chip for 2 min at 10 μl/min to test the response units (RU). All experiments were performed at least in triplicate.

3. Results and discussion

*3.1. Spectrophotometer and HPLC analysis of compounds (**1**–**5**) caging with HD1*

We execute multiple-caging strategy to cage with thrombin HD1 by using compounds (**1**–**5**) as previously described [20]. Each caging group was co-incubated with HD1 within 6–8 h respectively and then purified using Microcon YM-3 (3000 MW cutoff) centrifugal filters [6,15,38]. As shown in Fig. 2a, the spectrophotometric analysis exhibited two characteristic absorbance peaks of BHQ-HD1 and 7-HQ-HD1 in addition to main peak at 260 nm, whereas others showed none of the additional peaks. In general, the excess unattached compounds have been filtered and the characteristic absorbance peaks should attribute to the attachment of compounds. To further determine whether HD1 could be actually caged by BHQ-diazo and other compounds, reversed phase high performance liquid chromatography (RP-HPLC) method was performed. One part of BHQ-HD1 retained longer than their control counterparts, suggesting the binding of BHQ group with HD1. We consider that another elution profile similar as control HD1 was possibly caused by a heterogeneous product mixture of various degrees of caging. However, the elution peak of 6-HQ-HD1, 7-HQ-HD1 and 5-BHQ-HD1 only have a shoulder which probably means less conjugates between HD1 and caging group. 6-DBHQ-HD1 showed only single elution profile similar with control HD1 and therefore indicated the less reactive efficiency of these BHQ-diazo derivatives which alter the position of substituent to cage

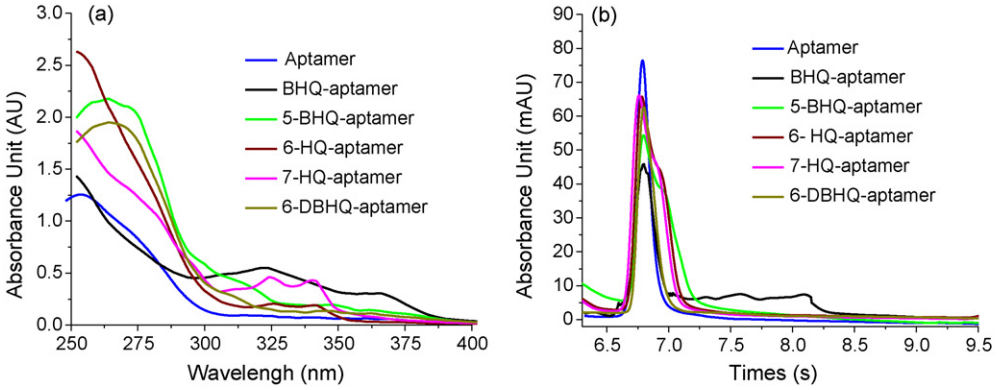

Fig. 2. Spectrophotometric (a) and RP-HPLC (b) analysis of compounds **1**–**5** caging with HD1.

with HD1 (Fig. 2b). Thus we considered that the position of bromine and hydroxyl on quinoline-based may play a key role in caging reaction. Whatever swapping the bromine substituent for other groups or exchanging the position of bromine or hydroxyl, the caging group was hardly to form a covalent bond with the phosphate moiety of the sugar-phosphate backbone of nucleic acid. Moreover, it should presume that the additional absorbance peak of 7-HQ-HD1 was caused by the higher self-absorbance of remainder unattached 7-HQ. After further purified by centrifugal filters, the additional peak subsequently disappeared (data not shown).

3.2. Spectrophotometric, gel electrophoresis and HPLC confirmation of photo-regulation of BHQ-HD1

Then, we further explore whether irradiation could release the HD1 from caging group as soon as possible. As shown in Fig. 3a, following illumination with 365 ± 5 nm ultraviolet light and purification, the additional peak of BHQ-HD1 disappeared and the photocleaved product resembled the control (non-caged) HD1. Likewise, RP-HPLC results indicate that upon exposure to light, the elution profile of the light-activated species resembled the elution profile of control HD1, suggesting the removal of the caging groups (Fig. 3b). The result of denaturing gel electrophoresis further confirms characteristic changes in mobility and intensity of band corresponding to the addition and removal of the BHQ caging groups (Fig. 3c). All the above results clearly demonstrated that BHQ-diazo could cage with HD1 through forming a covalent bond with the phosphate moiety of nucleic acid during simply co-incubation and compounds were subsequently released upon light-activation (Fig. 4).

3.3. RP-HPLC evaluation of photolysis sensitivity of BHQ-HD1

After confirming HD1 was caged by BHQ-diazo using none site-specific method, we subsequently examine the photolysis sensitivity of BHQ-HD1. Upon irradiation with 365 nm light, caged HD1 was released more than 90% within 30 s. This result indicates that the photolysis sensitivity of BHQ-HD1 improved 3-fold than Bhc at least which should contribute to lower cellular damage (Fig. 5).

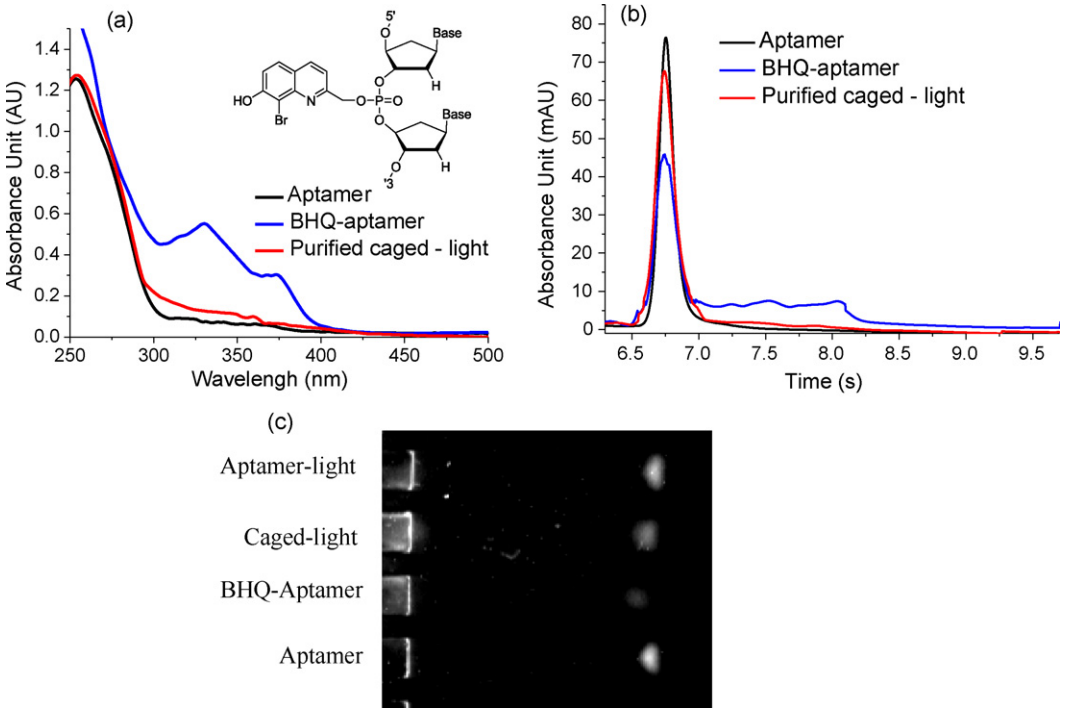

Fig. 3. Spectrophotometric (a) RP-HPLC (b) and gel electrophoresis (c) analysis of caged and light-activated HD1. All samples were processed in parallel. Inset of (a) shows the attachment of the BHQ to the phosphate backbone of HD1, considered to be the predominant site of caging.

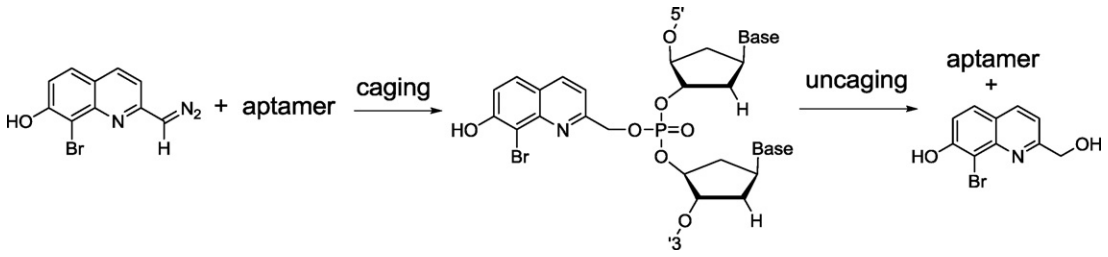

Fig. 4. Reaction of HD1 caged by BHQ-diazo.

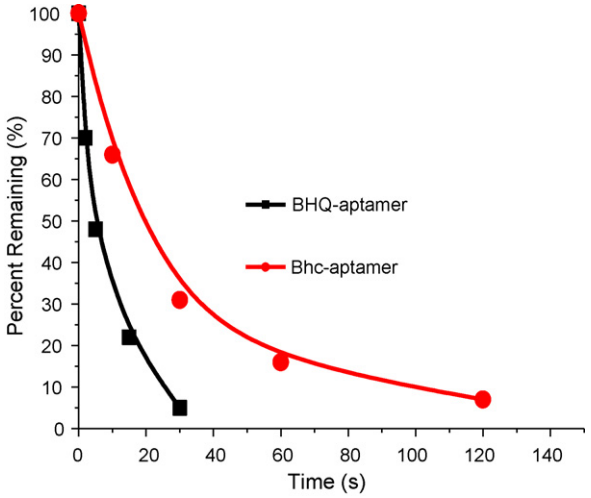

Fig. 5. Time-course of photolysis of BHQ-HD1, Bhc-HD1.

3.4. SPR measurements of caged HD1

Furthermore, the effect of the BHQ caging HD1 on the specific binding to target molecule was investigated by surface plasmon resonance (SPR) strategy [43]. We prefer to measure the fluctuation of affinity between HD1 and thrombin protein as our group previously reported [38]. As shown in Fig. 6, the response unit (RU) of caged HD1 decreased to 12% of control sample (named caging efficiency equal to 88%), and restored to more than 85% with subsequent photoirradiation within 30 s. The nonspecific binding between other oligonucleotides with thrombin can be eliminated. It was well known that the response unit is closely related with the specific affinity between HD1 and thrombin protein. This result clearly demonstrated that illumination ensured the caged HD1, which was encapsulated into an inactive form and lost affinity to target molecule, mostly recover to natural state and might act well to complete its natural function. Compared with Bhc caging (support information), BHQ exhibited bout 40% percent higher restoration (uncaging) efficiency. Likewise, the dissociation constants (K_D) of natural, caged and light-activated HD1 were performed under different concentrations with a steady state affinity method, and the result is 3.1×10^{-7} M, 6.3×10^{-6} M and 3.5×10^{-7} M, respectively. We have further optimized the caging and uncaging efficiency by systematically increasing the reactive equivalents of BHQ. For this experiment, 1- (25 μg/μl, 5 μl), 2- and 3-folds of BHQ-diazo relative to duplex were performed to cage target HD1 and result in the best effect on 2 equivalents of caging compound (support information). Besides, the caging efficiency of 6-HQ, 7-HQ and 5-BHQ analyzed by SPR was less than 35% which might partly caused by lower reactive efficiency (support information).

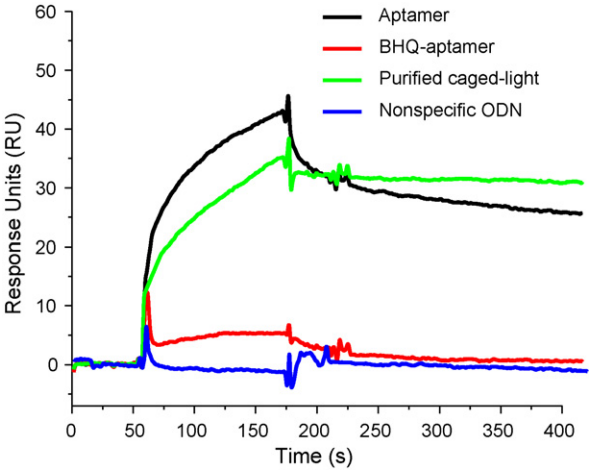

Fig. 6. SPR analyses of the affinity fluctuating of nature, caged and light-activated HD1.

As mentioned in pioneering work, the lower uncaging efficiency of this none site-specific caging method was due presumably to the difficulty of removing most of compounds with short irradiative period and thereby, acted as the primary obstacle to its further application [35]. Thus, we consider that the less amount of BHQ group for caging reaction and its higher photolysis sensitivity should primarily contribute to the improved uncaging efficiency.

4. Conclusion

In summary, BHQ-diazo (**1**) could photo-regulate HD1s activity during concise caging process and transitory photolysis period. It substantially improves the restoration efficiency and shows 3-fold photolysis sensitivity than previously reported Bhc group. We anticipate that the further development of higher photolysis sensitivity compounds for multiple caging with nucleic acid may provide a general strategy to improve uncaging efficiency and enlarge the application of this concise caging method to oligonucleotides. Moreover, better two-photon property of BHQ should contribute to potential photo-regulation of oligonucleotides's function under 2PE-mediated (two-photon excitation) with exact spatiotemporal resolution.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jphotochem.2010.02.009.

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