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RESEARCH ARTICLE

Both Bioorthogonal Ligations and Cleavages via Reactions of Chloroquinoxalines with *ortho*-Dithiophenols

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Abstract: Here we report a novel kind of bioorthogonal ligation and cleavage method via reactions of chloroquinoxalines (CQ) and *ortho*-dithiophenols (DT), in which double nucleophilic substitutions of *ortho*-dithiophenols to chloroquinoxalines provide the corresponding conjugates containing tetracyclic benzo[5,6][1,4]dithiino[2,3-*b*]quinoxaline with strong built-in fluorescence together with release of the other functional molecules. With this transformation, three cleavable linkers were designed and successfully used in release of the molecules containing biotin from the protein conjugates. The CQ-DT bioorthogonal reactions can be applied as the bioorthogonal ligations, bioorthogonal cleavages and trans-tagging of proteins, and show some advantages including readily accessible unnatural orthogonal groups, appealing reaction kinetics ($k_2 \approx 1.3$ $M^{-1}s^{-1}$), excellent biocompatibility of orthogonal groups and high stability of conjugates. This work provides an attractive complement to the previous bioorthogonal reaction toolbox and opens a new route for protein-fishing applications and in-gel fluorescence analysis.

Introduction

Bioorthogonal chemistry has become a powerful tool in chemical biology[1] (the concept of 'bioorthogonal chemistry' was first proposed by Bertozzi in 2003[2]), and it shows wide applications such as chemical labelling of biomolecules in living cells,[3] post-translational modification of proteins[4] and construction of antibody drug conjugates.[5] Therefore, the development of highly efficient bioorthogonal reactions is of great importance. The bioorthogonal reactions should meet the following conditions including fast rate, high yield, good solubleness and high stability of the reactants and product(s) under physiological conditions, non-toxicity to the biological system, minimizing steric interactions with the biomolecule and facilitating incorporation by the endogenous cellular machinery.[6] A number of bioorthogonal reactions including bioorthogonal ligations and cleavages have been developed thus far. The most representative bioorthogonal ligations include the native chemical ligation of proteins,[7] the protein labeling through the reaction of bisarsenical dyes with a genetically incorporated tetracysteine unit,[8] the biocompatible Staudinger ligation reaction of azides with modified triphenylphosphines,[9] the Cu(I)-catalyzed azide-alkyne cycloaddition,[10] the strain-promoted azide-alkyne cycloaddition,[11] the inverse electron-demand Diels-Alder (IED-DA) reaction,[12] and the boronate formation.[13] Compared with the diverse bioorthogonal ligations, the researches on the bioorthogonal cleavages are limited. The most representative examples include the light-induced bond cleavages,[14] the transition metal-catalyzed deallylation or depropargylation,[15] the specific IED-DA-induced 'click and release' reaction,[16] hydrazinolysis,[17] reduction of diazo[18] and disulfide compounds,[19] acid-induced hydrolysis of silyl esters.[20] Unfortunately, the conditions for some of bioorthogonal cleavages are harsh, which may affect the activity of biomolecules to some extent. In addition, the research of literatures shows that the reactions suitable for both the bioorthogonal ligations and cleavages are relatively rare. In 2013, Robillard and coworkers developed the drug decaging and release from antibodies through the tetrazine and *trans*-cyclooctene reaction, in which the IED-DA cycloaddition reaction initiated release of the drug attached to the *trans*-cyclooctene group (Figure 1a).[16a] The approach is probably the most popular reaction to date for the bioorthogonal ligation and release. Recently, Taran and co-workers also described an intresting bioorthogonal method via the reaction of iminosydnones and cyclooctynes allowing ligation and release of functional molecules (Figure 1b).[21]

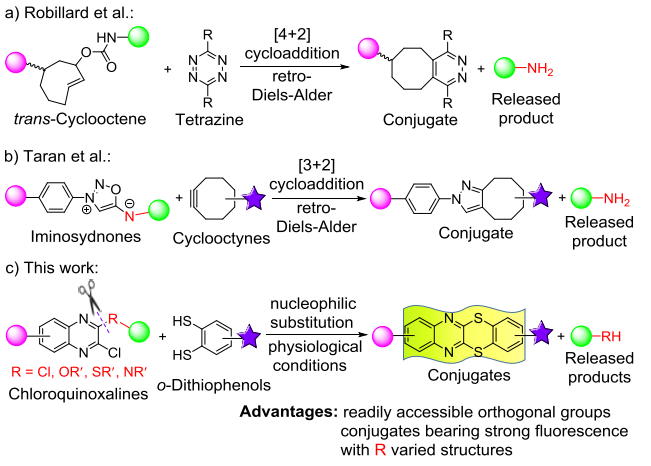

Figure 1. a) Robillard's click and release based on the tetrazine and *trans*-cyclooctene reaction. b) Taran's ligation and cleavage method via the reaction of iminosydnones with cyclooctynes. c) Our ligation and cleavage strategy via double nucleophilic substitutions of *o*-dithiophenols (DT) to chloroquinoxalines (CQ).

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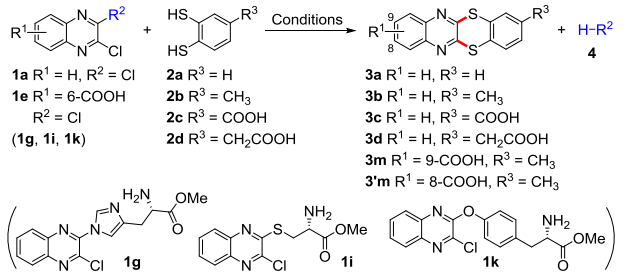
Herein, we report a novel and highly efficient strategy: both bioorthogonal ligations and cleavages via mild and selective reactions of readily accessible chloroquinoxalines (CQ) and *ortho*-dithiophenols (DT) (Figure 1c), in which double nucleophilic substitutions of *ortho*-dithiophenols to chloroquinoxalines provide the corresponding conjugates containing tetracyclic benzo[5,6][1,4]dithiino[2,3-*b*]quinoxaline with strong built-in fluorescence together with release of the other molecules.

***Table 1*:** Investigations on couplings of different substituted chloroquinoxalines (**1**) with *o*-dithiophenols (**2**).[a]



Entry	1	2	Condition[b]	3, Yield (%)
1	**1a**	**2a** (1 eq)	Condition A	**3a**, 94
2	**1a**	**2b** (1 eq)	Condition A	**3b**, 95
3	**1a**	**2c** (1 eq)	Condition B	**3c**, 97
4	**1a**	**2d** (1 eq)	Condition B	**3d**, 97
5	**1e**	**2b** (1 eq)	Condition B	**3m** + **3′m**, 98
6	**1e**	**2b** (1 eq)	Condition C	**3m** + **3′m**, >99
7	**1g**	**2d** (5 eq)	Condition C	**3d**, 96
8	**1i**	**2d** (10 eq)	Condition C	**3d**, 84
9	**1k**	**2d** (10 eq)	Condition C	**3d**, 98

[a] [**1**] = [**2**] = 50 mM for entries 1–5, isolated yields. [**1**] = 0.5 mM, [**2**] = 0.5–5 mM for entries 6–9, and the yields were determined by HPLC. [b] *Condition A*: K_2HPO_4 (2 eq), H_2O/DMF (1:4), room temperature (RT), 10 min. *Condition B*: K_2HPO_4 (3 eq), H_2O/DMF (1:4), RT, 10 min. *Condition C*: PBS (20 mM, pH 7.4)/DMF (1:1), RT, 60 min. See Tables S1–S7 for more screening details.

Results and Discussion

As mentioned above, it is highly desirable to develop a general and highly efficient method for bioorthogonal ligation and cleavage. We realized that reaction of *o*-dichloroquinoxalines with *o*-dithiophenols could be performed in organic solvents under the irradiation of light[22] or base-promoted conditions.[23] We surmised that the selective reactions of *o*-dichloroquinoxalines and their derivatives with *o*-dithiophenols could be used in bioorthogonal ligations and cleavages. Firstly, various chloroquinoxalines and *o*-dithiophenols were prepared (Schemes S1 and S2), and then couplings of chloroquinoxalines with *o*-dithiophenols were attempted in water/*N*,*N*-dimethylformamide (DMF) or phosphate buffer saline (PBS, pH 7.4)/DMF (Table 1). Wide investigations on the reagents and reaction conditions were performed, and some reactions of *o*-dichloroquinoxalines or mono-substituted chloroquinoxalines with *o*-dithiophenols readily occured in high yields with excellent selectivity (Tables S1–S7). As shown in Table 1, couplings of *o*-dichloroquinoxaline (**1a**) with four *o*-dithiophenols (**2a-2d**), respectively, in water/DMF provided conugates **3a**–**3d** within 10 min in 94–97% yields (entries 1–4). Subsequently, 2,3-dichloroquinoxaline-6-carboxylic acid (**1e**) was attempted as the partner of 4-methylbenzene-1,2-dithiol (**2b**), and an excellent yield (98%) was afforded (entry 5). We investigated reaction of **1e** with **2b** in PBS/DMF (entry 6), and the reaction also provided a high yield (> 99%). Inspired by the results above, we attempted to use various mono-substituted chloroquinoxalines as the partners of *o*-dithiophenols. Reactions of chloroquinoxalines **1g**, **1i** and **1k** containing histidine, cysteine and tyrosine units, respectively, with **2d** in PBS/DMF were surveyed, and the conjugate (**3d**) was obtained in 84–98% yields (entries 7–9). We found that the order of reactivity on the chloroquinoxalines containing three amino acid units was **1g** > **1k** > **1i**, and the yields obviously increased with ratio rise of **2d** (Table S7). Besides, reactions of chloroquinoxaline **1a** or **1k** with *o*-dithiophenol (**2d**) were peformed in cell growth medium (RPMI-1640, + 10% fetal bovine serum), respectively, and 2-13% efficiency loss was observed relative to the efficiency in PBS (Figure S8). Based on the results above, we think that couplings of *o*-dichloroquinoxalines or mono-substituted chloroquinoxalines with *o*-dithiophenols can be used as bioorthogonal ligations, and the reactions of mono-substituted chloroquinoxalines with *o*-dithiophenols provide opportunities for bioorthogonal cleavages, and simultaneous occurrence of bioorthogonal ligation and cleavage in one reaction.

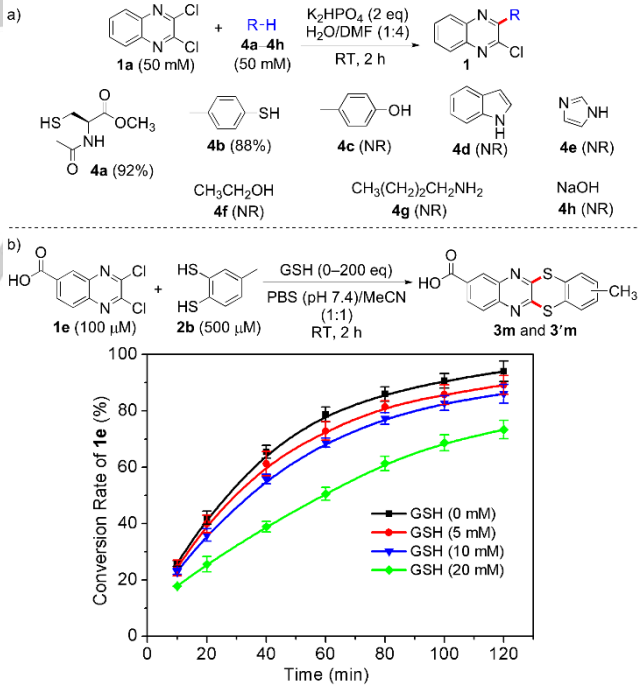


Figure 2. a) Investigation on reactivity of dichloroquinoxaline (**1a**) with different nucleophilic reagents. NR = No Reaction. b) Competitive reaction of **1e** with **2b** in the presence of GSH (0–200 equiv relative to **1e** and 0–40 equiv relative to **2b**). The conversion rates were determined by HPLC. The data are average from three replicate experiments.

To investigate the compatibility of orthogonal groups to versatile functional groups or complex systems, we first surveyed reactivity of *o*-dichloroquinoxaline (**1a**) with compounds **4a**–**4h** containing common functional groups including sulfydryl, hydroxyl,

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amino, indolyl and imidazolyl at millimolar concentration in water/DMF in the presence of two equiv of K_2HPO_4 for 2 h (Figure 2a). The experiments showed that only **4a** and **4b** containing sulfydryl could treat with **1a**, and no reaction occurred for phenol (**4c**), indole (**4d**), imidazole (**4e**), alcohol (**4f**), amine (**4g**) and even NaOH (**4h**). We found that **1a** was highly selective to thiols and tolerated a broad range of functional groups. Subsequently, reaction of **1a** with *N*-acetyl cysteine methyl ester (**4a**) was attempted in PBS (pH 7.4)/acetonitrile (v/v, 1:1), and different ratios of **4a**/**1a** (1:1, 5:1 and 10:1) led to conversion rates of 4%, 17% and 32% to mono-substituted chloroquinoxaline **1h**, respectively (Figure S5a). Although some treatment of *o*-dichloroquinoxalines with compounds containing sulfydryl such as cysteine derivative is disadvantageous to the bioorthogonal ligation, fortunately, the subsequent addition of *o*-dithiophenol can smoothly cleave the C-S bond to release free cysteine derivative, such as reaction of **1i** with **2d** (Table 1, entry 8). Next, the competitive reaction of **1e** with **2b** was investigated in the presence of different concentration of glutathione (GSH). The reaction efficiency suffered slight impact when GSH (0-10 mM) was 0–100 equiv relative to **1e** and 0–20 equiv relative to **2b**. However, the conversion rate decreased to 73% when GSH (20 mM) was 200 equiv relative to **1e** and 40 equiv relative to **2b** (Figures 2b and S6). In addition, we found that *o*-dithiophenols were relatively stable in cell medium in air for 12 h (Figure S9). One possible reason is that the formation of intramolecular hydrogen bond between two sulfydryls of *o*-dithiophenols inhibits their oxidation in air.

Rapid reaction of lower concentration of reactants is of great concern for modification of biomolecules under physiological conditions. Here, reaction of 2,3-dichloroquinoxaline-6-carboxylic acid (**1e**) with 4-methylbenzene-1,2-dithiol (**2b**) was used as the example to investigate the corresponding second-order rate constant in PBS (pH 7.4)/MeCN (Figure S11). The second order rate constant $k_2 \approx 1.3\pm0.1\ M^{-1}s^{-1}$ was calculated from the slope of the graph using the relationship: $k_{obs} = [\mathbf{2b}]k_2$.

UV-vis spectra of reactants **1e**, **2b** and their products **3m**, **3'm** were recorded, and the tetracyclic heteroacenes (**3m** and **3'm**) showed strong absorption peak at 398 nm. Importantly, the conjugates **3a**, **3c**, **3d**, **3m** and **3'm** with different substituents exhibited strong photoluminescence (PL) (Figure S12) in the visible light spectral region.[23]

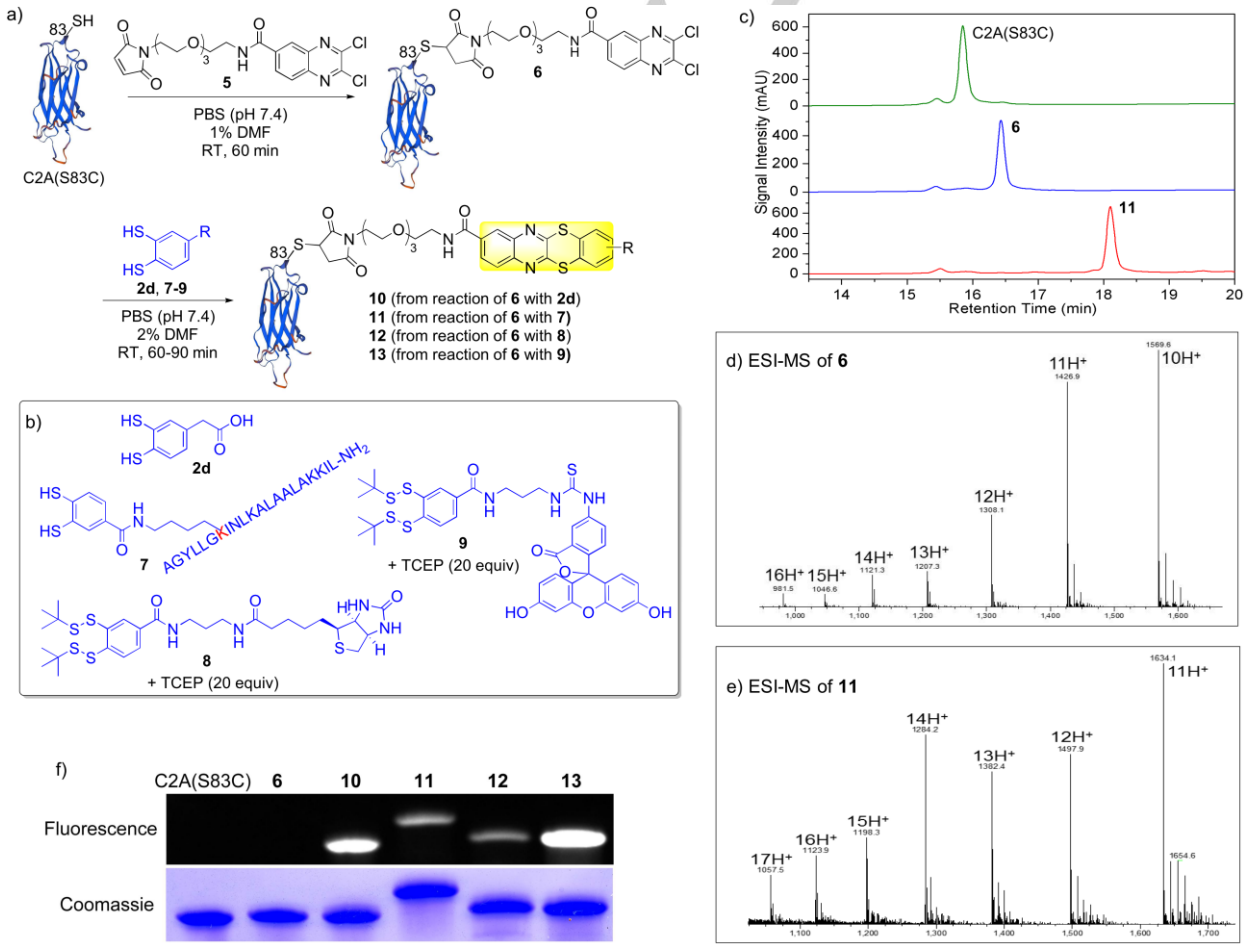

Figure 3. Bioorthogonal ligations of *o*-dichloroquinoxalines containing protein C2A(S83C) with different *o*-dithiophenols. a) Reaction routes. b) Four *o*-dithiophenols **2d**, **7**-**9**. Reaction conditions: C2A(S83C) (13 μM), **5** (65 μM), **2d** or **7**-**9** (130 μM), TCEP (2.6 mM), PBS (20 mM, pH 7.4) at room temperature for 60 or 90 min (*Note: The regioselectivity during formation of two C-S bonds is ignored*). c) HPLC traces of C2A(S83C), in-situ formed **6** and **11**. d) ESI-MS spectrum of **6** (Theoretical molecular weight: 15680.7. Calculated molecular weight: 15685.1). e) ESI-MS spectrum of **11** (Theoretical molecular weight: 17958.1. Calculated molecular weight: 17961.9). f) Gel analysis of C2A(S83C) (lane 1), *o*-dichloroquinoxaline functionalized C2A(S83C) **6** (lane 2) and bioorthogonal products **10**–**13** (lane 3–6). Fluorescence signals were acquired using EB channel (upper panel). Coomassie staining was used to assess protein loading (lower panel)

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Proteins are a kind of important biopolymers, and their chemical modification can provide a variety of purposes in chemical biology and medicine[24] such as installing a fluorophore[8,25] or binding partner[26] on proteins. Before investigating bioorthogonal reaction for protein modification, we first attempted to use GSH as a model peptide to appraise the CQ-DT reactions. The experiments showed that the reactions worked well in 60 min and almost quantitative conjugates were observed by HPLC and ESI-MS (Figures S13–S40). With these encouraging small molecules results in hand, we examined the CQ-DT reactions outlined above with a model protein to evaluate their potential as a novel bioorthogonal methodology. We chose an engineered variant of the C2A domain of Synaptotagmin-I, C2A(S83C) (molecular weight 15680 Da) with single and surface-exposed cysteine residue[27] as the model protein. As shown in Figure 3a, we first modified C2A(S83C) with maleimide-functionalized *o*-dichloroquinoxaline **5** to form **6** (HPLC and ESI-MS determination, see Figures 3c,d) via Michael addition of free cysteine residue in C2A(S83C) to maleimide group of **5**. To our delight, treatment of *o*-dichloroquinoxaline **6** containing protein C2A(S83C) with various *o*-dithiophenols (**2d**, **7**–**9**) (Figure 3b) in PBS (pH 7.4) at room temperature resulted in rapid and complete conversions of **6** to the corresponding ligation products (**10**–**13**) (Figures 3a, S47–S51) via analysis of ESI-MS (such as Figure 3e), HPLC (such as Figure 3c) and gel analysis (Figure 3f). It is noteworthy that *o*-dithiophenols containing cell transmembrane peptide (**7**),[28] biotin (**8**) and fluorescein (**9**) all provided complete conversions (*Note: tBuS-deprotection was carried out with tris(2-carboxyethyl)phosphine (TCEP) in PBS buffer for tBuS-protected o-dithiophenols*).

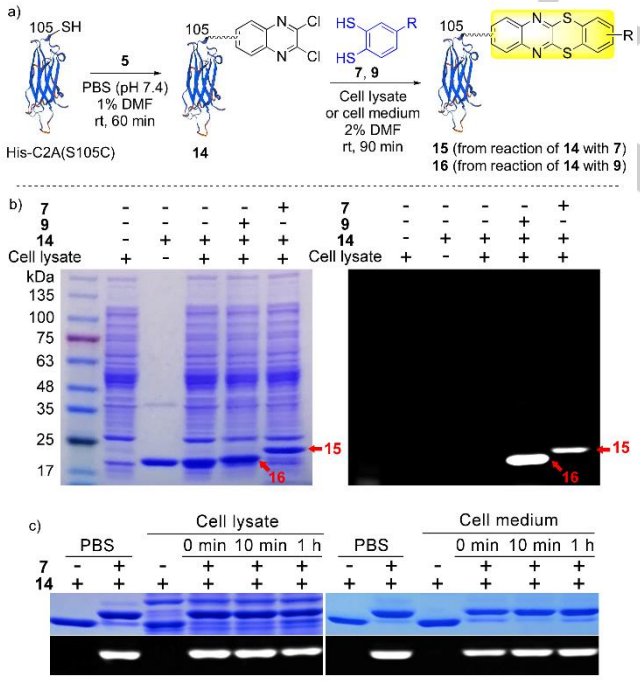

Figure 4. Efficiency in a crude cell lysate or cell medium on the CQ-DT reactions. a) Reaction routes. b) Gel analysis of the CQ-DT conjugates in the cell lysate. c) *o*-Dichloroquinoxaline-functionalized His-C2A(S105C) **14** was incubated in cell lysate or cell medium for the indicated time, then treated with *o*-dithiophenol **7**, and the conjugates were determined by Gel analysis. Fluorescence signals were acquired using EB channel.

To evaluate efficiency of the CQ-DT reactions in a more complex system, we investigated conjugation of *o*-dithiophenols containing cell transmembrane peptide (**7**) or fluorescein (**9**) with *o*-dichloroquinoxaline-functionalized His-C2A(S105C) (**14**) (***14*** *was prepared via Michael addition of sulfydryl of cysteine residue on His-tagged C2A(S105C) to maleimide-functionalized chloroquinoxaline* ***5***) in *E. coli* cell lysate (Figure 4b). The experimental results showed that the reactions proceeded smoothly and were almost not interfered by free thiol in the vicinity of complex aqueous system, and the modified His-C2A(S105C) protein could be visualize by fluorescence imaging at concentration of around 15 μM through the CQ-DT reactions in cell lysate. Importantly, the reactions exhibited remarkable selectivity without detectable background signals for fluorescence labeling in the cell lysate. In addition, *o*-dichloroquinoxaline-functionalized His-C2A(S105C) **14** was incubated in cell lysate or cell medium for the indicated time, then treated with *o*-dithiophenol **7**, and the conjugates were determined by Gel analysis. We found that more than 80% efficiency was maintained in cell lysate or cell growth medium relative to PBS (Figure 4c and Figure S52). Therefore, the CQ-DT reactions are compatible with different biomolecules and can be used for protein labeling in PBS, cell lysate or cell growth medium. We believe that the conjugates containing tetracyclic benzo[5,6][1,4]dithiino[2,3-*b*]quinoxaline with built-in fluorescence will provide important applications for antibody-free Western Blot analysis.

Furthermore, we investigated practicability of the CQ-DT reactions as versatile cleavable methods for protein-fishing applications. The previous conditions for the desorption of the strong biotin-avidin affinity usually are harsh such as heating, which cause the unspecific release of background proteins through random adsorption to the avidin surface, and the fact hampers the subsequent analysis. Therefore, it is of significance to develop an efficient method for gentle release of biotin-protein complexes.[1e] Encouraged by our results above, we here investigated bioorthogonal ligations and cleavages using pre-targeted model protein bearing a biotin moiety. Our strategy is shown in Figure 5a: Michael addition of sulfydryl of cysteine residue on the protein to maleimide-functionalized chloroquinoxaline (**I**) linking with biotin leads to **II**, then treatment of **II** with 2-(3,4-dimercaptophenyl)acetic acid (**2d**) provides ligation product **III** and cleavage product **IV**, and the obtained conjugates are analyzed by gel analysis and ESI-MS. We designed and synthesized three maleimide-functionalized chloroquinoxalines with cleavable linkers, and their coupling with C2A(S83C) afforded pre-targeted proteins **17**–**19**. Firstly, chloroquinoxaline derivative **17** containing C2A(S83C) and a biotin moiety through linkage of thiol ether was used for the bioorthogonal ligation and cleavage. Incubation of **17** with **2d** in PBS (pH 7.4) at room temperature for 90 min provided conjugate **20** in almost quantitative yield (Figure 5b) via analysis of HPLC, ESI-MS (Figure 5e) and gel analysis (Figure 5f) releasing cleavage product **21**. Next, treatment of previously prepared chloroquinoxaline derivative **18** containing C2A(S83C) and a biotin moiety through linkage of 4-(hydroxymethyl)phenol with **2d** under the similar conditions gave **20** in almost quantitative yield (Figure 5c) via analysis of HPLC, ESI-MS and gel analysis releasing **22**, in which formation of **22** underwent a cascade process including cleavage of a C-O bond and

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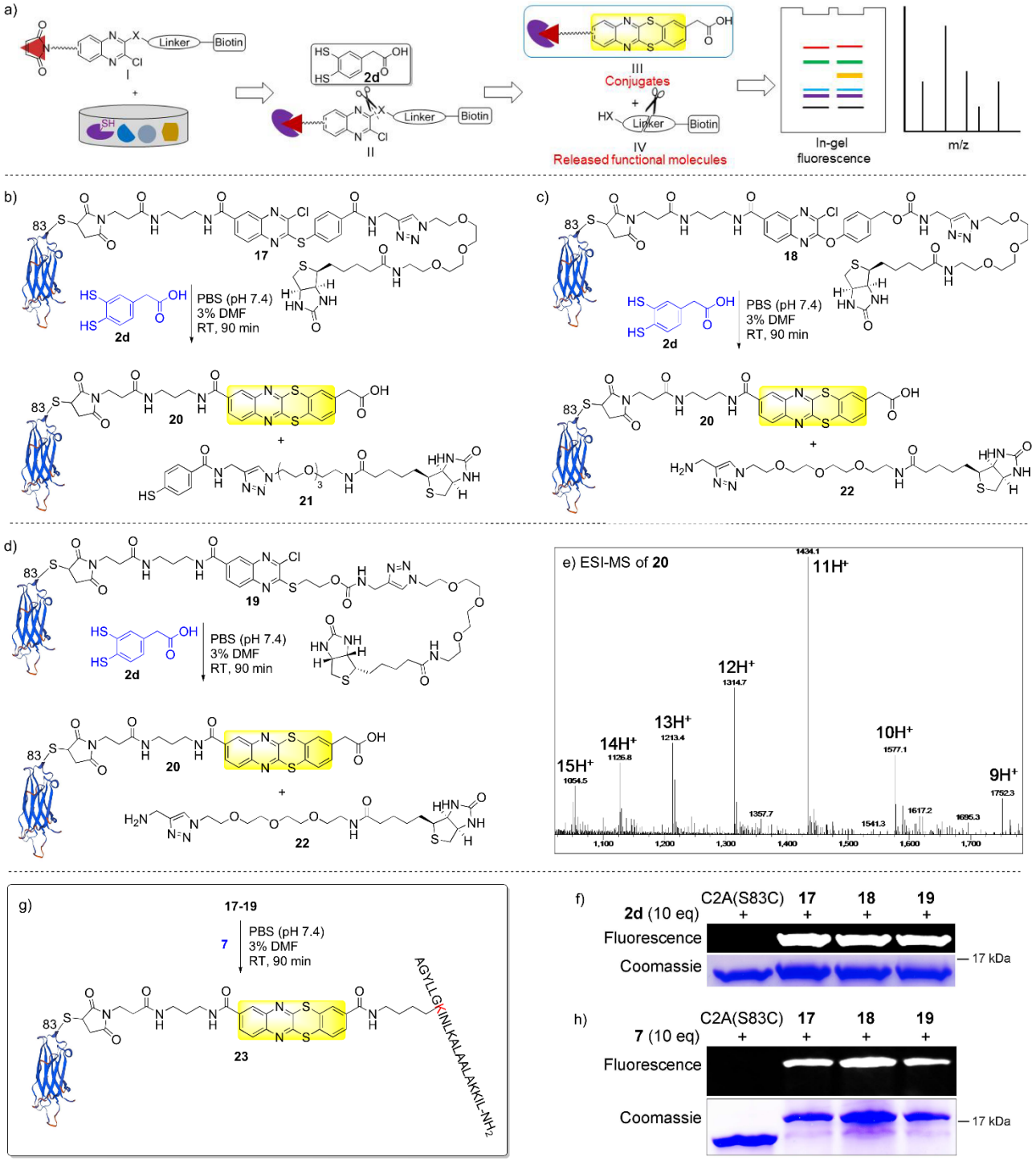

Figure 5. Both bioorthogonal ligations and cleavages. a) Our strategy on biorthogonal ligations and cleavages. b) Reaction of chloroquinoxaline derivative **17** with **2d**. c) Reaction of chloroquinoxaline derivative **18** with **2d**. d) Reaction of chloroquinoxaline derivative **19** with **2d**. Reaction conditions: **17**–**19** (13 μM), **2d** (130 μM), PBS (10 mM, pH 7.4) at room temperature for 90 min. e) ESI-MS of **20**. f) Gel analysis of C2A(S83C) (lane 1) and **20** (lanes 2–4 from reactions of **17**–**19** with **2d**, respectively). g) Reactions of **17**–**19** with **7**. Reaction conditions: **17**–**19** (13 μM), **7** (130 μM), PBS (10 mM, pH 7.4) at room temperature for 90 min. h) Gel analysis of C2A(S83C) (lane 1) and **23** (lanes 2–4 from reactions of **17**–**19** with **7**, respectively). Fluorescence signals were acquired using EB channel (upper panel). Coomassie staining was used to assess protein loading (lower panel). (*Note: The regioselectivity during formation of two C-S bonds is ignored.*)

release of an functional molecule (it means a decaged process).[29] The decaged process was confirmed through reaction of **S80** with **2b** in PBS (pH 7.4)/acetonitrile (v/v, 1:1) at room temperature (Figure S54a). Further, we made chloroquinoxaline derivative **19** containing C2A(S83C) and a biotin moiety through linkage of 2-mercaptoethanol, and reaction of **19** with **2d** also led to a complete conversion of **19** to ligation product **20** releasing **22** via a cascade process including cleavage of a C-S bond and release of an functional molecule[30] (Figure 5d). The decaged process was also verified through reaction of **S81** with **2b** in PBS (pH 7.4)/acetonitrile

(v/v, 1:1) at room temperature (Figure S54b). Therefore, the experiments in Figures 5b-5d and S54 showed that the present method could be used in release of biologically active molecules. Finally, we attempted reactions of different substituted chloroquinoxalines **17–19** containing C2A(S83C) with **12**, and conjugate **23** was observed in almost quantitative yields (Figure 5g) determined by HPLC, ESI-MS and gel analysis (Figure 5h). Therefore, the present method provides a novel strategy for bioorthogonal ligations and cleavages, and it will be widely applied in chemical labelling of biomolecules, purification or enrichment of chemically modified proteins, construction of antibody drug conjugates and other fields.

Conclusion

In summary, we have developed a novel kind of bioorthogonal reactions of readily available chloroquinoxalines (CQ) and *ortho*-dithiophenols (DT) incorporated with functional molecules such as amino acids, biotin, fluorescein, peptides and protein, and the corresponding conjugates containing tetracyclic benzo[5,6][1,4]dithiino[2,3-*b*]quinoxaline were formed in excellent yields in organic solvents, PBS buffer, cell medium, or cell lysate together with release of the other functional molecules in direct or indirect ways. The obtained conjugates are highly stable in the cell medium (Figure S10) and show strong fluorescence. The built-in fluorescence avoids active alteration of biomolecules for secondary derivatization with a fluorophore, which is very useful for probe development and screening. The CQ-DT bioorthogonal reactions can be used as the bioorthogonal ligations, bioorthogonal cleavages and the trans-tagging of proteins under physiological conditions. We believe that the CQ-DT bioorthogonal reactions with multifunctions of bioorthogonal ligations, cleavages and built-in fluorescence should provide a new strategy for bioorthogonal chemistry and will find a wide range of applications.

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Keywords: bioorthogonal ligations • bioorthogonal cleavages • chloroquinoxalines • *ortho*-dithiophenols • protein modification

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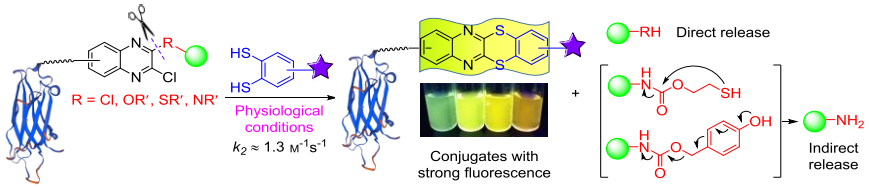

Bioorthogonal reactions of chloroquinoxalines (CQ) with *ortho*-dithiophenols (DT) under the physiological conditions provided corresponding conjugates with strong built-in fluorescence releasing functional molecules with direct or indirect manners. With this transformation, three cleavable linkers were designed and successfully used in release of the molecules containing biotin from the protein conjugates