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Photoreductive Uncaging of Fluorophore in Response to Protein Oligomers by Templated Reaction *in Vitro* and *in Cellulo*

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S Supporting Information

ABSTRACT: The photoreduction of azide-based immolative linker by Ru(II) conjugates to uncage rhodamine was achieved using different oligomeric protein templates. The generality of the approach was validated with three sets of ligand having varying affinity to their target (biotin, desthiobiotin and raloxifene). The reaction rates of the templated reaction was found to be at least 30-fold faster than the untemplated reaction providing a clear fluorescent signal in response to the protein oligomer within 30 min. The templated reaction was found to also proceed *in cellulo* and could be used to identify acetyl coenzyme A carboxylase (ACC) in *Pseudomonas aeruginosa* and human cell lines as well the and estrogen receptor (ER).

Fluorescent probes designed to interrogate protein localization and function are a cornerstone of live cell imaging and a powerful tool for biological investigations.[1] Interrogation of protein homo- or heterodimerization has generally been achieved through the use of two interacting fluorophores leading to a FRET (Förster resonance energy transfer).[2] Herein we report an alternative strategy leveraged on a chemical reaction promoted by the high effective concentration achieved upon ligands binding (Figure 1A). The strategy uses a pair of ligands derivatized, individually, with suitable reactive partners. At low concentration, the bi-molecular reaction is slow; however, upon ligand binding, the reactive partners are brought into close proximity thereby increasing their effective concentration and accelerating the reaction. This process is analogous to nucleic acid templated reaction whereby hybridization aligns compatible reagents.[3−5] As in the latter case, a potential advantage of this strategy over a simple FRET readout is that the system can be anticipated to turn over multiple substrates leading to signal amplification. More importantly, no genetically encoded constructs are required to interrogate protein interactions. The azide functionality has been demonstrated to be highly biorthogonal[6] and suitable to mask the fluorescence of pro-fluorophore[7−16] in DNA or RNA-templated reactions with phosphine probes. We have reported an azide-reduction triggered immolative linker which is broadly applicable to uncage bioactive small molecules and fluorophores.[10] More recently, we have shown[9] that DNA-templated

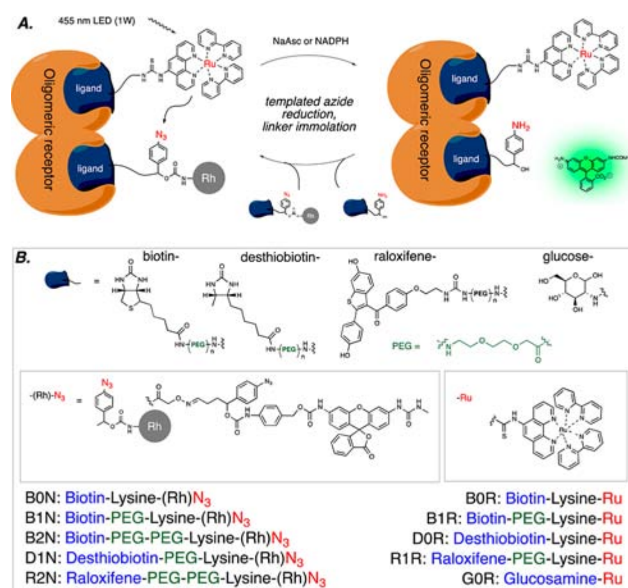

Figure 1. (A) Schematic representation of protein templated reaction. (B) Structure of ligands and conjugates used.

linker cleavage can also be achieved photoreductively with catalytic ruthenium complex in the presence of sodium ascorbate (NaAsc).[17]

To evaluate the templated photoreductive cleavage of the azide linker using proteins as templates, we first focused on streptavidin as a target based on the fact that it forms a tetramer with two binding sites in close proximity. Furthermore, the strong interaction with biotin should ensure that the ligand−protein interaction is sufficiently long-lived for the reaction to proceed. While the distance between the two binding sites is 18.8 Å (Figure S1), bis-biotin based ligand cannot bind linearly due to the protein structure. It has been shown that the optimal distance between two biotin groups in the bis-biotin ligand is ~31 Å and that the steric properties of the linker is important.[18] With these considerations, biotin derivatives with various PEG (polyethylene glycol) units between the ligand and azide linker having lengths of 18, 30, and 42 Å, respectively (B0N−B2N,

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Figure 1B) were prepared. Similarly, two biotin ruthenium conjugates (B0R and B1R) with 10 and 22 Å linkers were prepared. The fact that the reaction leads to uncaging of rhodamine fluorophore offers a convenient method to follow the progress of the reaction in real time. Irradiation with a 1 W LED lamp (455 nm) of both reaction partners at 1 μM afforded undetectable reaction. In the presence of 1 equiv of streptavidin, the best combination (B0N+B1R, Figure 2) afforded a reaction that was faster than the reaction with 20 mM of phosphine (10^4 higher concentration). Interestingly, comparing the kinetics of the different combinations clearly suggest that there is an optimal linker length. If the linkers are too short for the reagent to interact efficiently (B0N+B0R), the rate of reaction was 30 times slower (taking the slope of the reaction after 30 min as an approximation of pseudo-first-order rate). If the linkers are too long (B1N+B1R; B2N+B1R, B2N +B0R), the reaction does proceed but at 4 times slower rate than the optimal length reflecting the poorer preorganization of the reagents (lower effective concentration). However, the position of the spacer is not critical and B1N+B0R had a comparable rate to B0N+B1R. While these results were encouraging, the quasi-irreversible interaction of biotin ($K_d \approx$ 10 fM) with streptavidin is exceptional and not extrapolative to more common small molecule-protein interaction. We next used desthiobiotin conjugates as ligands. Desthiobiotin is a truncated analogue of biotin with an affinity of 330 nM for streptavidin[19] and hence more representative of prototypical small molecule protein interactions. Gratifyingly, the reaction was found to precede equally well with this lower affinity interaction. While the interaction of biotin−streptavidin does not allow turnover due to its exceptionally slow dissociation, we reasoned that the weaker binding of desthiobiotin should allow ligand exchange, and hence, the protein could act catalytically. Indeed, using 20% and 10% of streptavidin afforded 64% and 38% conversion, respectively, after 2 h suggesting that streptavidin acted catalytically (Figure 3). Furthermore, the reaction could be performed with only 20% of the ruthenium conjugate D0R and streptavidin.

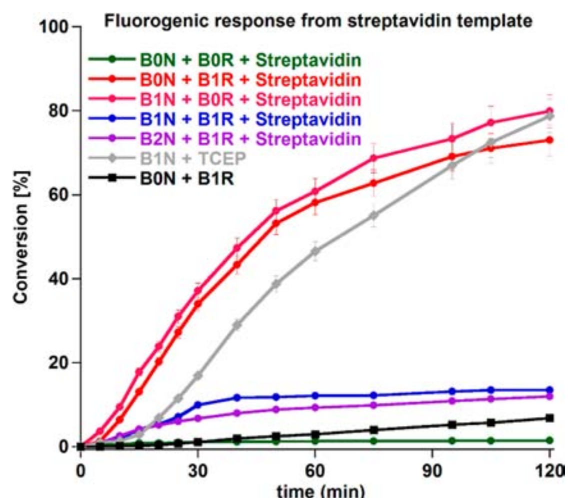

Figure 2. Kinetics of fluorescence enhancement for streptavidin template due to the released rhodamine from biotin based azide probes in the presence of Ru(II) probes. Medium: 500 mM Tris-HCl buffer (pH 7.4) containing 0.1% formamide; conc. of B0N, B1N, B2N, B0R, B1R and streptavidin, 1.0 μM each; conc. of NaAsc, 10 mM, and TCEP, 20 mM at room temperature.

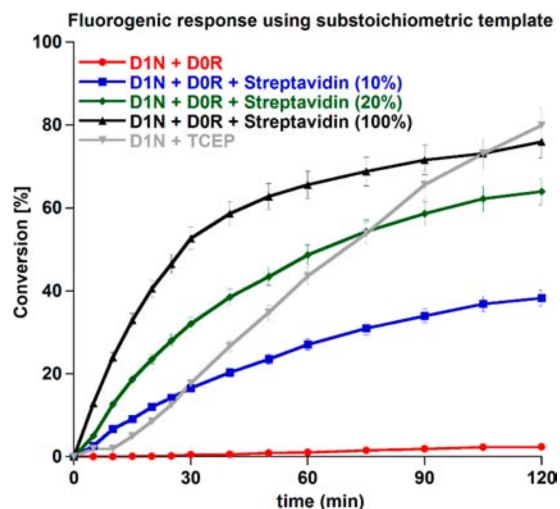

Figure 3. Kinetics of fluorescence enhancement for streptavidin template due to the released rhodamine from desthiobiotin based probe in the presence of Ru(II) probe. Medium: 500 mM Tris-HCl buffer (pH 7.4) containing 0.1% formamide; conc. of D1N, D0R, 1.0 μM each; conc. of NaAsc, 10 mM, and TCEP, 20 mM at room temperature.

To assess the generality of this concept, we next investigated another target protein, the estrogen receptor (ER). It is a homodimer and has successfully been targeted by covalent dimeric ligands.[20] A crystal structure of ER with raloxifene (RAL) indicated a distance of 34.7 Å (Figure S3) that should be compatible with the templated reaction using R2N and R1R ligands. As with streptavidin, a dramatic rate acceleration was observed for the photoreduction of R2N by R1R in the presence of the ER compared to the same reaction without the protein (Figure 4). Furthermore, substitution of the raloxifene−ruthenium conjugate by glucosamine−ruthenium conjugate reduced the rate of reaction by 25-fold (taking the slope after 30 min as an approximation of the pseudo-first-order rate constant) further establishing that binding of both raloxifene

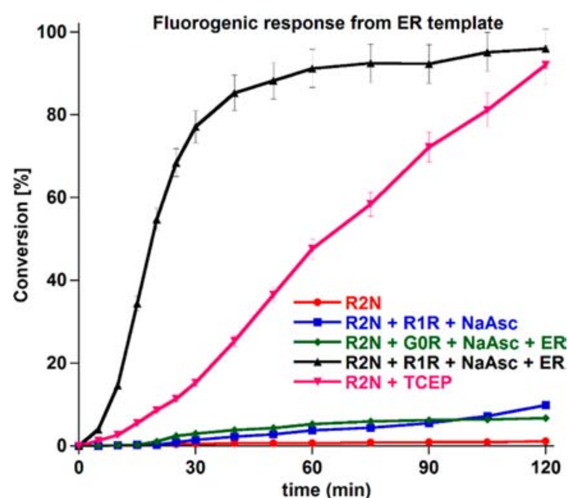

Figure 4. Kinetics of fluorescence enhancement for ER template due to the released rhodamine from raloxifene based R2N probe in the presence of two Ru(II) probes R1R and G0R at room temperature. Medium: 500 mM Tris-HCl buffer (pH 7.4) containing 0.1% formamide; conc. of R2N, R1R, G0R and ER, 300 nM each; conc. of NaAsc, 10 mM, and TCEP, 20 mM.

derivatized ligands (R2N and R1R) is necessary for the reaction to proceed.

Next, we asked whether this reaction could be performed in a cellular context. In bacteria, acetyl CoA carboxylase (ACC) is a multicomponent protein that utilizes biotin as a cofactor. The distance between the biotin-binding domains is 26.91 Å (Figure S4) suggesting that it could be addressed with the templated chemistry. We used *Pseudomonas aeruginosa* (PA01) bacteria, pathogenic bacteria devoid of streptavidin, to assess the performance of the reaction. Measuring bulk fluorescence indicated that the best ligand combination was B0N with B1R (shorter and longer linkers performed worse) and that the increase in fluorescence was 2.5-fold higher for the best probe than a control experiment with B0N+G0R (Figure S5). While the reaction *in vitro* requires a stoichiometric reducing agent (NaAsc), the results obtained with live bacteria suggested that cellular environment was sufficiently reductive for the reaction to proceed. The applicability of the protein templated reaction to fluorescence imaging was then investigated. After treatment with B0N and B1R, the bacteria were irradiated with the 455 nm LED lamp (1 W) for 30 min. Fluorescence imaging study revealed that the fluorogenicity was associated with live bacteria (Figure 5) and clearly discernable from a negative control experiment using B0N and G0R. The quantitative analysis of the images indicated 8-fold higher fluorescence (Table S1) in the bacteria treated with biotin probes (B0N+B1R) relatively to the control with biotin and glucose amine (B0N+G0R).

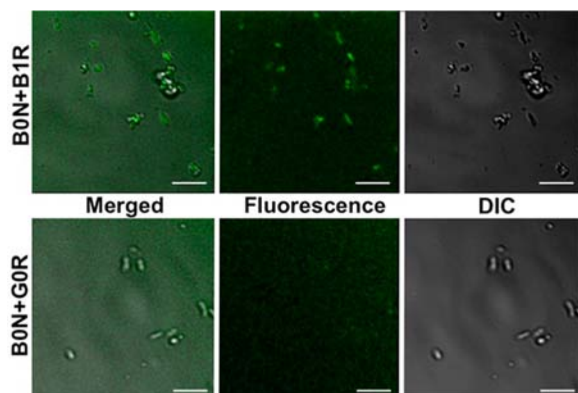

Figure 5. Fluorescence imaging of live PA01 bacteria after the treatment of biotin based probes B0N and B1R (top) and control experiment with B0N and G0R (bottom). The medium along with live bacteria was irradiated with 455 nm LED (1 W) for 30 min before imaging. The bacteria were excited at 460−500 nm for rhodamine. Scale bar: 5 μm.

We then turned our attention to human cell lines. ACC_{α} has been found to be upregulated in HER2-driven oncogenic cell lines and was reported to be significantly more abundant in BT-474 relative to MCF-7.[21] On the basis of this observation, we asked whether the templated reaction with biotin could discriminate between these two different cell lines. Treatment with the optimal probes (B0N+B1R) followed by irradiation with 455 nm LED showed a higher intensity of BT474 than MCF7 (Figure 6). On the other hand, MCF-7 is known to express high level of ER.[22] Incubation of MCF7 with R2N and R1R indeed afforded high fluorescence. In contrast, the same experiment with the glucose-ruthenium conjugate (G0R) in lieu of the raloxifene−ruthenium conjugate (R1R) afforded significantly less fluorescence (Figure 7). Taken together, the data supports that the observed fluorescence is the product of the proposed templated reaction and that the photo cleavage of the azide based linker can be achieved in live cell without addition of external reducing agents in response to a protein oligomer template.

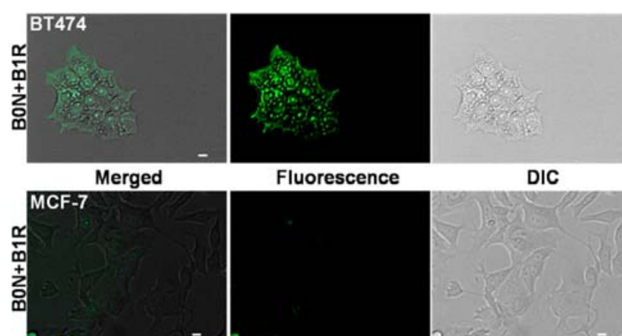

Figure 6. Fluorogenic imaging after the treatment of biotin based probes B0N and B1R in live BT474 cells (top) and in live MCF-7 cells (bottom). The live cells were irradiated with 455 nm LED (1 W) for 30 min before imaging. The cells were excited at 460−500 nm for rhodamine. Scale bar: 20 μm.

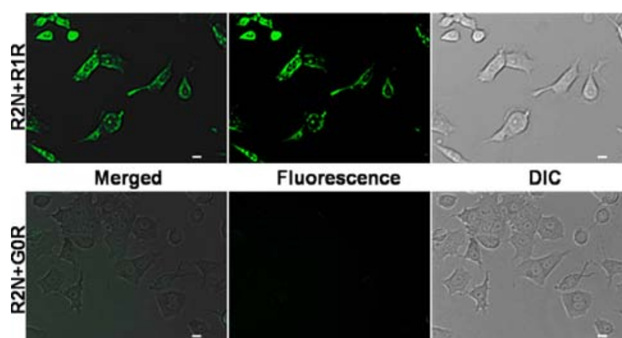

Figure 7. Fluorescence imaging of live human breast cancer MCF-7 cells after the treatment of raloxifene based probes R2N and R1R (top) and control experiment with R2N and G0R (bottom). The medium, along with live cells, was irradiated with 455 nm LED (1 W) for 30 min before imaging. The cells were excited at 460−500 nm for rhodamine. Scale bar: 20 μm.

In conclusion, we have tailored the azide-based immolative linker for protein templated photoreduction with Ru(II) conjugates. Strong discrimination between the template and untemplated reaction (over 30-fold) is observed after 30 min reaction making this approach suitable for imaging. We demonstrated the applicability of the templated reaction to image oligomeric receptors *in cellulo*. While this study focused on homodimeric or oligomeric targets, the approach should lend itself to heterodimeric or oligomeric interactions as well. Though the linker was used strictly to uncage a rhodamine fluorophore in the present study, this linker is broadly applicable to uncage bioactive small molecules and pharmaceutical compounds, which would be released in a target specific manner. To the best of our knowledge, this is the first report of a templated reaction that can report on protein interactions.

ASSOCIATED CONTENT

Supporting Information

Experimental procedures, full structure of all compounds used, and supporting figures and table. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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