Live-Cell Imaging

DOI: 10.1002/ange.200903233

Fast and Sensitive Pretargeted Labeling of Cancer Cells through a Tetrazine/*trans*-Cyclooctene Cycloaddition**

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There is considerable interest in the use of bioorthogonal covalent chemistry, such as "click" reactions, to label small molecules located on live or fixed cells.[1] Such labeling has been used for the visualization of glycans, activity-based protein profiling, the site-specific tagging of proteins, the detection of DNA and RNA synthesis, investigation of the fate of small molecules in plants, and the detection of posttranslational modification in proteins.[2–4] Most reported applications rely on either the copper-catalyzed azide–alkyne cycloaddition, which is limited to in vitro application owing to the cytotoxicity of copper, or the elegant strain-promoted azide–alkyne cycloaddition, which is suitable for live-cell and in vivo application but is hindered by relatively slow kinetics and the often difficult synthesis of cyclooctyne derivatives.[4,5] New bioorthogonal reactions that do not require a catalyst and show rapid kinetics are therefore of interest for different molecular-imaging applications at the cellular level. Herein we demonstrate the use of an inverse-electron-demand Diels–Alder cycloaddition between a serum-stable 1,2,4,5-tetrazine and a highly strained *trans*-cyclooctene to covalently label live cells. We applied this reaction to the pretargeted labeling of epidermal growth factor receptor (EGFR) tagged with cetuximab (Erbitux) on A549 cancer cells. We found that the tetrazine cycloaddition to *trans*-cyclooctene-labeled cells is fast and can be amplified by increasing the loading of the dienophile on the antibody. This highly sensitive targeting strategy can be used to label proteins by treatment with a secondary agent at nanomolar concentrations for short durations of time.

Recently, we and others explored strain-promoted inverse-electron-demand Diels–Alder cycloaddition reactions of 1,2,4,5-tetrazines for bioconjugation.[6,7] We showed that the cycloaddition of a tetrazine with a norbornene can be applied to the pretargeted imaging of live breast cancer cells. However, the rate of cycloaddition of the tetrazine with norbornene was $1.6\,\text{M}^{-1}\text{s}^{-1}$ in serum at 20 °C. This rate is comparable to previously reported rates for optimized azide–cyclooctyne cycloaddition reactions and requires micromolar concentrations for sufficient labeling.[3,4] On the basis of previously reported rate constants, we decided to investigate the coupling of tetrazines with more-strained dienophiles.[8] Higher rate constants would enable faster and more efficient labeling. Thus, less of the labeling agent would be required, and the background signal would be decreased.

Fox and co-workers recently reported the use of a highly strained *trans*-cyclooctene for bioconjugation.[6,9] Although the rates reported were impressive, the tetrazine that yielded the fastest rate has limited stability to nucleophiles and aqueous media, with significant degradation observed after several hours. In contrast, we reported the use of the novel asymmetric tetrazine **1**, which is very stable in water as well as in whole serum: a prerequisite for in vivo applications.[7] We hypothesized that tetrazine **1** would react with *trans*-cyclooctene significantly faster than the previously reported norbornene and this would greatly improve the sensitivity of cell labeling by tetrazine cycloaddition.

With this goal in mind, we synthesized the *trans*-cyclooctene dienophile **2** in two steps from a commercially available cyclooctene epoxide. The *trans*-cyclooctene reacted readily with tetrazine **1** to form isomeric dihydropyrazine conjugation products in greater than 95% yield (Figure 1a; see also the Supporting Information). The *trans*-cyclooctenol **2** can be converted into the reactive succinimidyl carbonate, and the carbonate can be conjugated to amine-containing biomolecules, such as monoclonal antibodies, through the formation of a carbamate linkage. To determine the second-order rate constant for the reaction of the tetrazine with the *trans*-cyclooctene, we modified surface arrays of *trans*-cyclooctene-functionalized antibodies with a fluorescent tetrazine probe and monitored the fluorescence signal over time (see Figure S3a in the Supporting Information). From these data, we determined a second-order rate constant of $6000 \pm 200\,\text{M}^{-1}\text{s}^{-1}$ at 37 °C (see Figure S3b in the Supporting Information). This rate constant is several orders of magnitude higher than the previously reported value for the cycloaddition of tetrazine **1** with a norbornene derivative, as well as the previously reported rate constants for bioorthogonal click reactions used to label live cells covalently.[3,4,7]

To demonstrate the utility of the reaction of a tetrazine with a *trans*-cyclooctene for live-cell imaging, we chose to label EGFR expressed on A549 lung cancer cells with the anti-EGFR monoclonal antibody cetuximab. The concept of pretargeting is illustrated in Figure 1b. The multistep labeling of monoclonal antibodies is of interest as a result of the long blood half-life of antibodies. This property leads to poor target-to-background ratios when the antibodies are labeled directly with imaging agents or cytotoxins.[10] A small-

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[**] We thank Dr. Ned Keliher for helpful advice. This research was supported in part by NIH grants U01-HL080731 and T32-CA79443.

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.200903233.

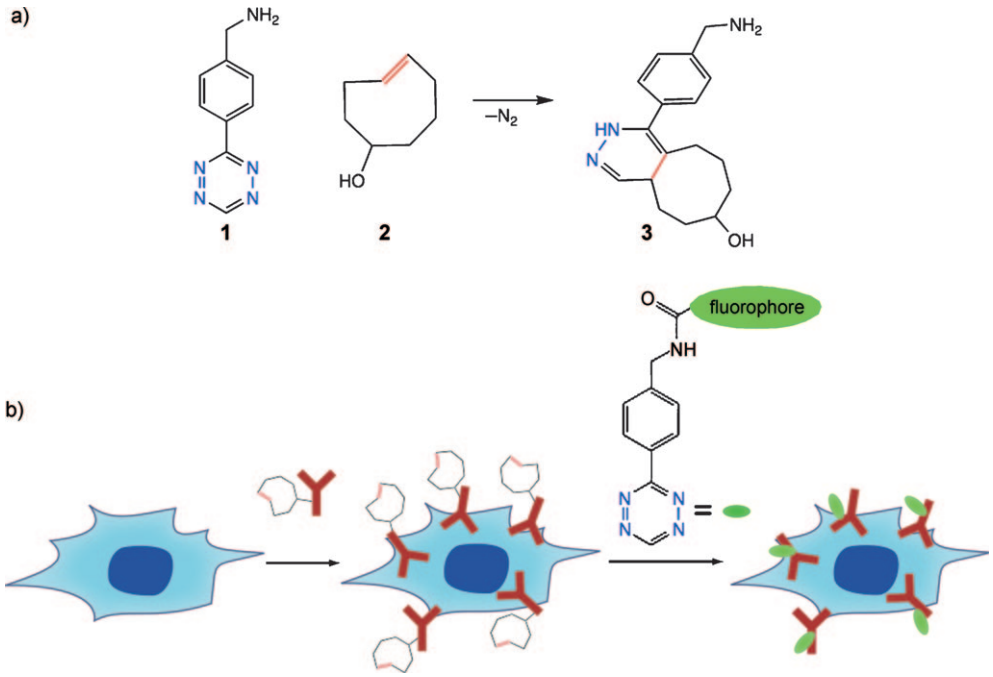

Figure 1. a) Reaction of benzylaminotetrazine **1** with *trans*-cyclooctenol **2** by an inverse-electron-demand Diels–Alder cycloaddition. Dinitrogen is released, and dihydropyrazine coupling products, such as **3**, are formed. b) Live-cell pretargeting. Cancer cells (blue), which overexpress EGFR, are exposed to the cetuximab–*trans*-cyclooctene conjugate (red). In the next step, the pretargeted cells are labeled with a tetrazine bearing a fluorophore such as VT680 (green).

molecule-based pretargeting strategy that relies on irreversible covalent chemistry may circumvent these problems and find general application for the delivery of imaging agents and therapeutics.[11]

For cell-labeling studies, we chose an anti-EGFR antibody as our target, as EGFR is of central importance in cancer-cell signaling[12] and therefore a key target for therapeutic inhibition,[13] and because prior studies with fluorophore-labeled antibodies would serve as a reference.[14] Commercially available cetuximab was labeled with *trans*-cyclooctene succinimidyl carbonate and used for pretargeting experiments. We chose to work with a green fluorescent protein (GFP) positive A549 lung cancer cell line that has been shown to have upregulated levels of EGFR.[15] To label cells pretargeted with *trans*-cyclooctene-bearing antibodies, the tetrazine amine **1** was conjugated to a commercially available far-red indocyanine fluorophore, Vivo-Tag 680 (VT680 purchased from VisEn Medical). The decision to use tetrazine–fluorophore probes was based on the commercial availability of numerous amine-reactive fluorophores. Furthermore, we had used this compound previously and shown that the tetrazine moiety is serum-stable and reacts rapidly with strained dienophiles.[7]

Initial pretargeting experiments used cetuximab modified by both *trans*-cyclooctene and a single Alexa Fluor 555 dye (AF555 purchased from Invitrogen). The AF555 dye was conjugated to the antibody in addition to the *trans*-cyclooctene to track the location of the antibody and determine the specificity of sequential VT680–tetrazine labeling. A549 cancer cells were first incubated with cetuximab–*trans*-cyclooctene/AF555 (100 nM) for 45 minutes in serum. The cells were then washed, incubated at 37 °C with tetrazine–VT680 (500 nM) for 10 min in 100 % fetal bovine serum (FBS), washed again, and imaged immediately by confocal microscopy (Figure 2). Direct labeling of the antibodies with AF555 was monitored in the red channel (Figure 2b, red). The antibody was clearly visible both on the surface of the cells and inside the cells as a result of EGFR internalization.[16,17] Covalently bound tetrazine–VT680 could be visualized clearly in the near-infrared (NIR) channel (Figure 2c, green). Merging of the red and NIR channels revealed excellent colocalization of the AF555 and VT680 signals with little background fluorescence. This result indicates that the reaction of the tetrazine is extremely selective. As expected, the reaction occurred primarily on the surface of the cells, where EGFR concentrations are highest. A smaller amount of cell-internalized, vesicle-associated NIR fluorescence was

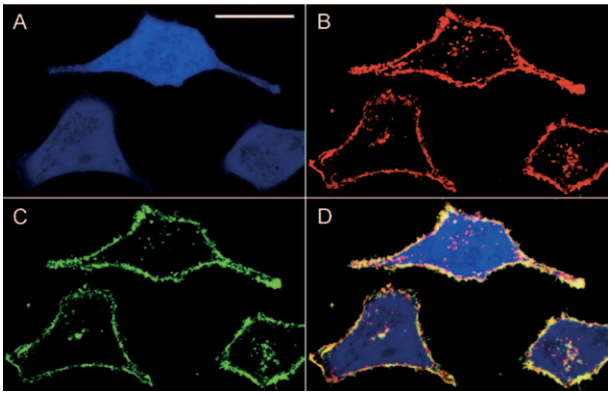

Figure 2. Confocal microscopy of cetuximab-pretargeted GFP-positive A549 lung cancer cells after tetrazine–fluorophore labeling. A) GFP channel. Scale bar: 30 μm. B) Red channel: Cetuximab–*trans*-cyclooctene antibodies were also labeled with AF555 directly and imaged in the rhodamine channel. Some of the antibody has been internalized, as indicated by the signal inside the cells. C) Near-IR channel showing the location of bound tetrazine–VT680 probe (500 nM, 10 min, 100% FBS, 37 °C). D) Merging of GFP, red, and near-IR channels. The red and near-IR channels show excellent colocalization, especially at the surface of the cells. Intracellular cetuximab that has not reacted with tetrazine–VT680 is clearly visible and was probably internalized prior to the addition of the extracellular tetrazine–VT680 probe.

also observed. This fluorescence is probably a result of EGFR internalization after treatment with tetrazine–VT680.[16,17] Control experiments with either unlabeled cetuximab and tetrazine–VT680 or *trans*-cyclooctene–cetuximab and unlabeled VT680 resulted in no NIR fluorescence (see Figure S4 in the Supporting Information).

Next, we tested whether labeling could be observed without a washing step to remove the probe. The desire to avoid such a step is relevant to applications in which one is unable to perform stringent and multiple washing steps, such as intracellular labeling, experiments in which cell handling has to be minimized (with rare cells or highly specialized cells), and in vivo labeling. The concentration of the tetrazine–VT680 label was lowered to 50 nM to enable observation of the covalent modification in real time. The images in Figure 3 were taken during continuous imaging of

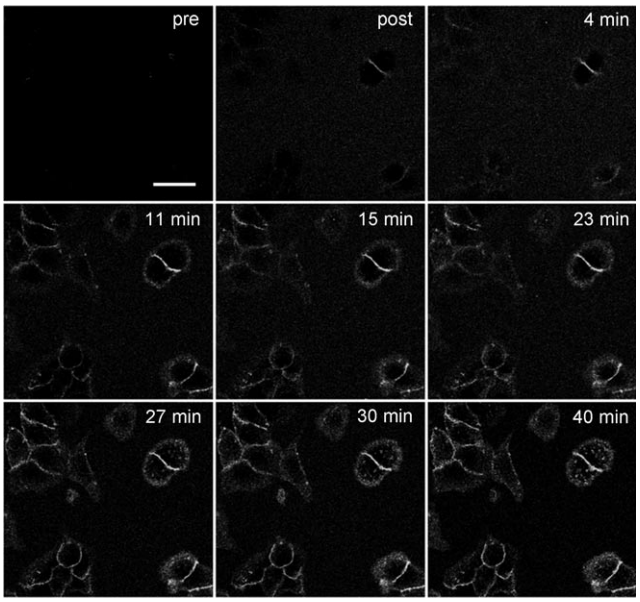

Figure 3. Real-time imaging of the tetrazine labeling of pretargeted A549 cells. Cells were exposed to cetuximab–*trans*-cyclooctene, washed, and imaged in 100% FBS by using the near-IR channel (top left). The FBS was removed and immediately replaced with FBS containing tetrazine–VT680 (50 nM; top middle image). Images were taken periodically over 40 min. The signal around the cell surfaces continues to increase as a function of time. Scale bar (top left): 30 μm.

the cycloaddition of the tetrazine–VT680 to the pretargeted *trans*-cyclooctene on live cancer cells in 100% FBS. Tetrazine–VT680 first became visible as it reacted and concentrated on the surface of cells; at later times, punctate spots within the cell were visible as tetrazine-labeled cetuximab was internalized.

In an attempt to improve the signal-to-background ratio, we increased the loading density of the reactive *trans*-cyclooctene on the targeted antibodies. A greater number of reactive sites per antibody should lead to more fluorophore per antibody after labeling and thus result in signal amplification. To vary the *trans*-cyclooctene loading, we exposed cetuximab to different molar excesses of the amine-reactive *trans*-cyclooctene. The conjugates were modified with tetrazine–VT680, and the resulting fluorochrome absorbance was used to estimate the number of reactive *trans*-cyclooctene units per antibody. In this fashion, cetuximab bearing one, three, five, and six tetrazine–VT680-reactive *trans*-cyclooctene moieties were prepared. Owing to the large size of indocyanine dyes, for the higher loadings, the number of reactive *trans*-cyclooctene moieties is probably lower than the actual number of *trans*-cyclooctene moieties on the antibody. These *trans*-cyclooctene–cetuximab conjugates bound to EGFR-expressing A549 cells with excellent stability (see Figure S5 in the Supporting Information).

We used flow cytometry to gain a more quantitative understanding of live-cell fluorescent labeling with the tetrazine. A549 cells were incubated with cetuximab (50 nM) modified with either zero, one, three, five, or six reactive *trans*-cyclooctene units. The cycloaddition was carried out with tetrazine–VT680 (500 nM) at 37 °C in 100% FBS. After 30 min, the cells were washed, and the fluorescence intensity was analyzed by flow cytometry. Figure 4a shows the relative VT680 signal intensity after 30 min for all five loadings of the *trans*-cyclooctene. To illustrate the practical effect of this

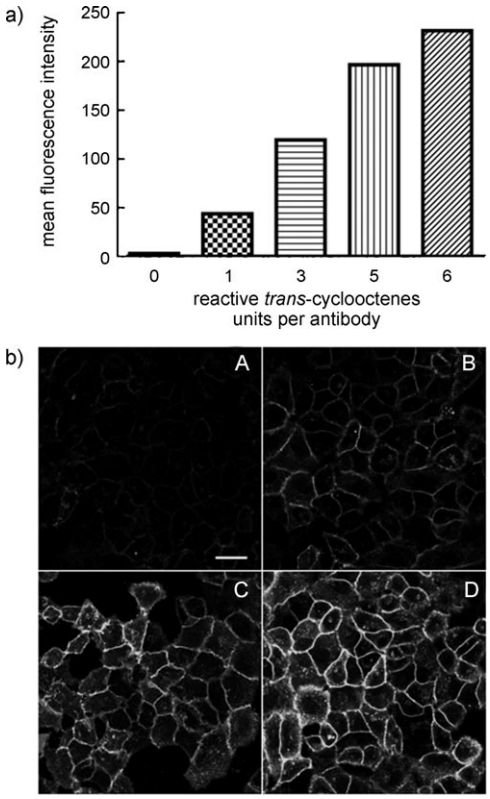

Figure 4. a) Analysis of live-cell labeling by flow cytometry. Fluorescence intensity after treatment for 30 min with tetrazine–VT680 (500 nM) versus the loading of reactive *trans*-cyclooctene on the antibody. b) Confocal microscopy of A549 cells pretargeted with cetuximab loaded with A) one, B) three, C) five, or D) six *trans*-cyclooctene moieties and then labeled by treatment with tetrazine–VT680 (100 nM) for 10 min. The increasing fluorescence signal correlates with the increasing level of labeling of the antibody with the *trans*-cyclooctene. (All cells were exposed to the antibody in the same concentration.) Scale bar (top left): 30 μm.

amplification on the imaging of live cells, we pretargeted A549 cells with cetuximab (100 nM) conjugated to one, three, five, and six reactive *trans*-cyclooctene moieties, then exposed the cells to tetrazine–VT680 (100 nM) for 10 min prior to imaging with confocal microscopy (Figure 4b). Cells pretargeted with *trans*-cyclooctene-conjugated cetuximab constructs with higher loadings of the *trans*-cyclooctene were visualized readily, and the signal diminished as the amount of the dienophile on the antibody decreased. The ability to amplify signals by loading increased amounts of a small molecule on the antibody could provide a strategy for increasing the signal-to-background ratio for in vivo pretargeting schemes.

In conclusion, we have developed a highly sensitive technique for the covalent labeling of live cancer cells on the basis of the cycloaddition of a tetrazine to a highly strained *trans*-cyclooctene. With the appropriate choice of a cell-permeable labeling agent, this method should be readily extendable to intracellular labeling and could facilitate the tracking of tagged small-molecule drugs, signaling proteins, or other components of the cellular machinery within live cells. Furthermore, it may be possible to incorporate tetrazines and *trans*-cyclooctene-substituted nonnatural amino acids into proteins of interest in a site-specific manner and then to reveal them in live cells. Given its speed and sensitivity in whole serum, this labeling reaction should be adaptable to in vivo imaging applications.

Received: June 15, 2009
Published online: August 20, 2009

Keywords: bioorthogonal reactions · cancer · cycloaddition · imaging agents · tetrazines

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