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## Metabolic Remodeling of Bacterial Surfaces via Tetrazine Ligations

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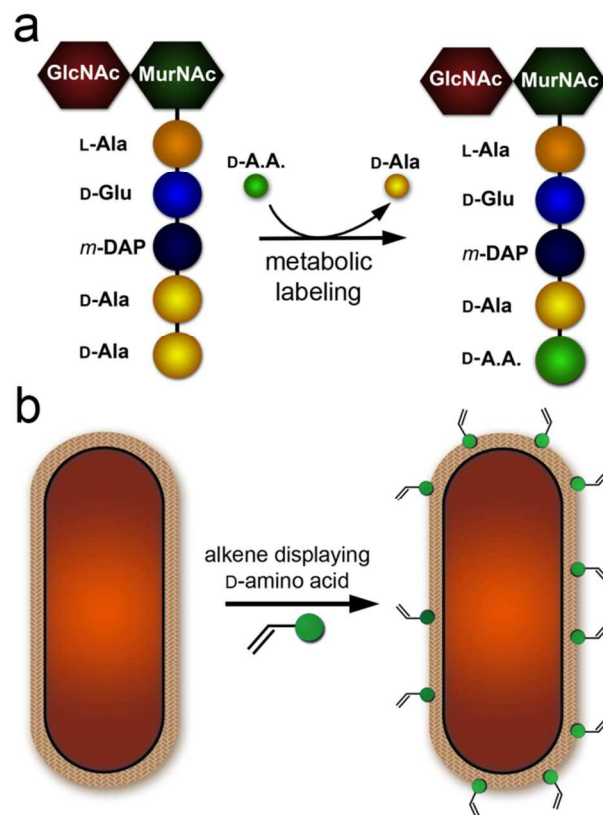
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**Bioorthogonal click ligations are extensively used for the introduction of functional groups in biological systems. Tetrazine ligations are attractive in that they are catalyst-free and display favorable kinetics. We describe the efficient remodeling of bacterial cell surfaces using unnatural D-amino acids derivatized with tetrazine ligation handles. The metabolic incorporation of these unnatural D-amino acids onto bacterial cell surfaces resulted in a site-selective installation of fluorophores.**

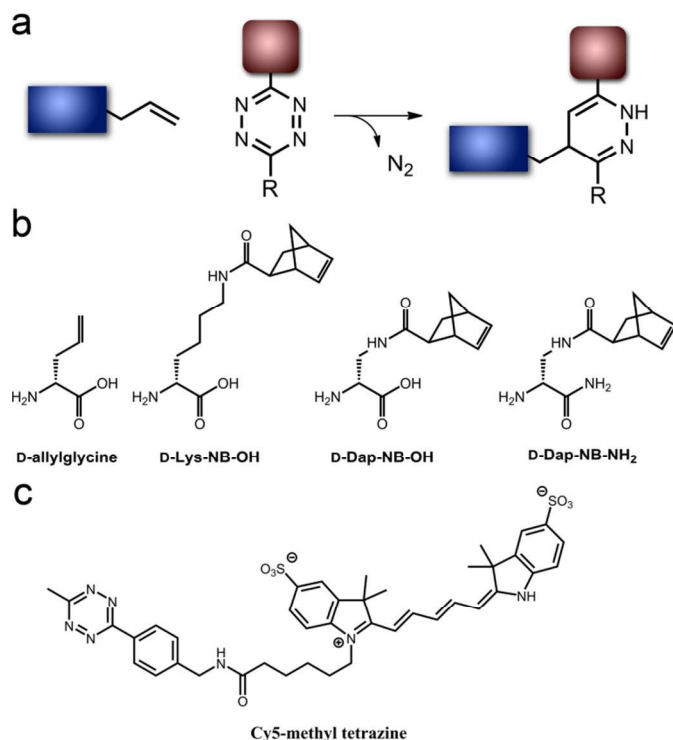
Bioorthogonal chemical reactions have proven to be indispensable tools for probing and monitoring many biological processes with minimum sample interference.<sup>1-4</sup> These reactions have the advantage of being compatible with biological conditions (aqueous medium and physiological temperatures), typically induce minimal cytotoxicity, and display excellent selectivity. To date, a number of ligation strategies have been developed that are widely utilized.<sup>5</sup> One of the areas that has advanced the most since the introduction of bioorthogonal ligation reactions is the field of bio-imaging.<sup>6, 7</sup> The ability to introduce tags that illuminate the localization and movement of biomacromolecules has completely revolutionized the way biological processes are monitored.<sup>4</sup> In this communication, we demonstrate for the first time the metabolic site-selective fluorescent labeling of bacterial cell surfaces using tetrazine-based ligation.

All bacteria are surrounded by a protective cell wall, where the major structural component is peptidoglycan.<sup>8</sup> Bacterial peptidoglycan is vital to all known bacteria, as it provides resistance to unfavorable external conditions and counteracts internal osmotic pressure. The polymeric peptidoglycan resides on the exterior surface of bacterial cell membranes of Gram-positive organisms (inner membranes for Gram-negative organisms).<sup>9, 10</sup> It is composed of repetitive sugar units linked to short oligopeptide chains. Of note, the oligopeptide unit contains several D-amino acids, a distinctive characteristic of bacteria. Nascent peptidoglycan is loaded onto the existing structure by penicillin binding proteins (PBPs). PBPs are a major class of bacterial enzymes that are important for bacterial growth and division.<sup>11-13</sup> In particular, transpeptidase domains of PBPs introduce crosslinks between neighboring oligopeptide strands by removing the terminal D-alanine to form an acyl intermediate.



**Scheme 1** (A) Schematic diagram of the swapping of exogenous D-amino acid with terminal D-Alanine. (B) Metabolic incorporation decorates the bacterial cell surface with tetrazine ligations.

The PBP-anchored intermediate can be captured by a nearby nucleophilic *meso*-diaminopimelic acid (*m*-DAP) or L-lysine residue, resulting in the formation of a covalent crosslink. Recently, it was demonstrated that unnatural D-amino acids from the surrounding medium can also displace the acyl-intermediate, thus resulting in the swapping of the terminal D-alanine with unnatural D-

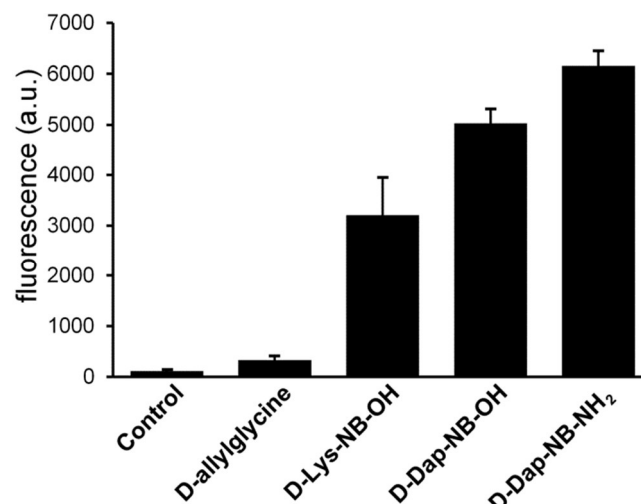


**Fig. 1** (a) Tetrazine ligation. The boxes represent conjugated species to the tetrazine handles. (b) Chemical structure of 4 unnatural alkenyl displaying D-amino acids. (c) Chemical structure of Cy5-methyl tetrazine.

amino acids (Scheme 1).<sup>14-23</sup> Metabolic swapping with exogenous D-amino acids is a facile method for remodeling bacterial cell surfaces.

The site-selective remodeling of bacterial surfaces can be a powerful way to introduce small epitopes or entirely non-native biomacromolecules.<sup>24</sup> Remodeled surfaces can, in turn, be leveraged for interrogation of endogenous biological processes (e.g., surface binding) or as potential therapeutic interventions. We recently exploited this methodology to induce the recruitment of endogenous antibodies to the surface of various bacteria, including the human pathogen *Staphylococcus aureus* (*S. aureus*), using unnatural D-amino acids conjugated to small antigenic epitopes.<sup>25, 26</sup> Although unnatural D-amino acids represent a novel and promising strategy to decorate bacterial cell surfaces, lack of tolerability for large amino acid sidechains may prevent its wider utilization. During our development of antibody-recruiting D-amino acids, we observed a severe reduction in incorporation with increasing size of the sidechain of the D-amino acid. We propose to decouple the two components (surface modifications and epitope/macromolecule installation) by using a two-step process.

In this second-generation remodeling strategy, the unnatural D-amino acid delivers a small biorthogonal handle to the bacterial cell surface with the goal of optimizing incorporation efficiency (Scheme 1). The remodeled cell surface is subsequently exposed to the complementary ligation handle to afford a covalent linkage. A major advantage of using biorthogonal chemistry is that it should permit the introduction of larger molecules onto the cell surface, which may otherwise be prohibitive using a one-step strategy. D-amino acids displaying alkyne or azido handles on the side chain have been established as viable two-step methods of installing fluorophores onto bacterial peptidoglycans using copper-catalyzed click reactions.<sup>17, 18, 27</sup> The Bertozzi laboratory has recently demonstrated



**Fig. 2** Flow cytometry analysis of tetrazine ligation on the surface of *S. aureus*. Cells were labeled overnight in the presence of unnatural D-amino acid variants or in media alone. Tetrazine reaction was then performed with Cy5-methyl tetrazine. Data are represented as mean + SD (n=3).

the feasibility of using strain-promoted click chemistry to fluorescently label bacterial cell surfaces.<sup>19, 20</sup> We identified tetrazine ligation as a prime candidate to implement our two-step strategy with the ultimate goal of efficiently installing highly antigenic molecules (some of which cannot be achieved using the one-step method) on bacterial cell surfaces *in vivo*.

Tetrazine ligation is a relatively new method in the repertoire of click reactions yet it has already attracted considerable attention due to the combination of its small size, fast kinetics, and established *in vivo* compatibility.<sup>28-31</sup> The reaction proceeds through an inverse-electron demand Diels-Alder reaction, releasing innocuous nitrogen gas as a byproduct (Fig. 1). Herein, we show for the first time that bacterial cell surfaces can be selectively remodeled using tetrazine click chemistry *via* transpeptidase mediated incorporation of D-amino acids. Most importantly, we show that this strategy affords live-cell peptidoglycan labeling of the human pathogen *S. aureus*.

We initially set out to probe the promiscuity of transpeptidase to tolerate D-amino acids derivatized with alkene functional groups. Previously, we had observed that a number of unnatural side chains could be accommodated in the swapping of exogenous D-amino acids with surface anchored terminal D-alanine. Yet, the incorporation efficiency appears to be highly dependent on the structure of the sidechain. A small panel of D-amino acids were synthesized using standard solid phase chemistry to probe (1) the structure of the alkene for tetrazine ligation, (2) transpeptidase restraints in the size/flexibility of the amino acid side chain, and (3) alkene displaying unnaturally C-terminated D-amino acids (Fig. 1, ESI†). Tetrazine ligation on bacterial cell surfaces was performed by overnight incubation of *S. aureus* in the presence of each unnatural D-amino acid variant. We chose to investigate ligations using Gram-positive *S. aureus* due to its prominent pathogenicity and potential for future application of this technology for immuno-modulation.<sup>32</sup> Successful incorporation of the D-amino acid leads to the covalent installation of alkene functional groups at the peptidoglycan (Scheme 1). For the optimization stage of our study, cells were fixed, treated with Cy5-methyl tetrazine, and fluorescence labeling was measured via flow cytometry.

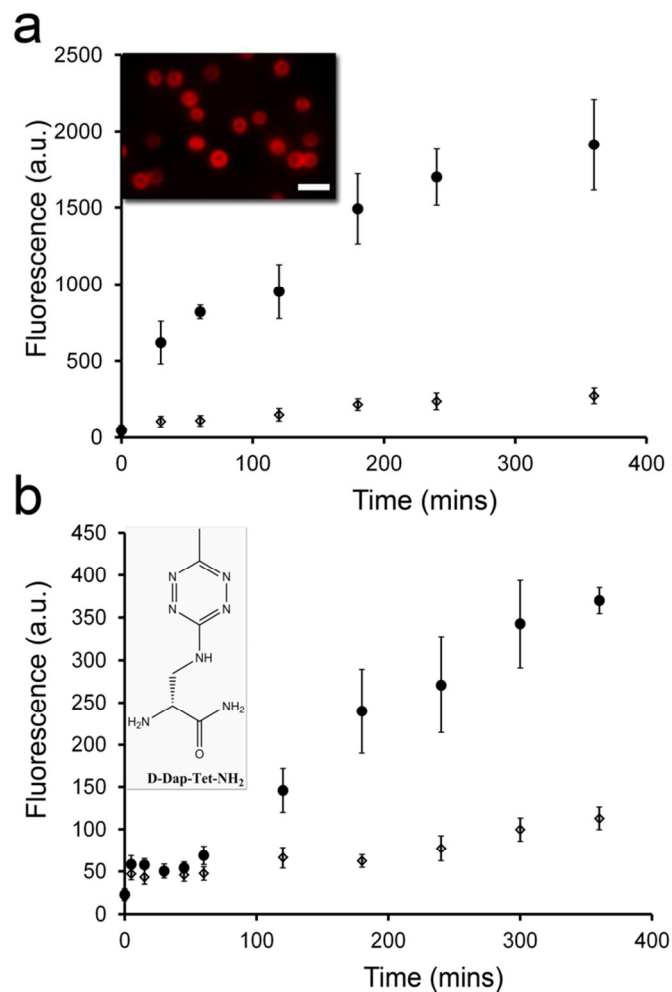
At first, we evaluated two unnatural D-amino acids for their compatibility with our strategy: D-allylglycine and D-Lys-NB-OH.

D-allylglycine was chosen due to the small sidechain size and previously reported compatibility of this alkene configuration with tetrazine ligations.<sup>33</sup> The small sidechain is expected to lead to higher incorporation levels. D-Lys-NB-OH was built by conjugating a norbornene group to the  $\epsilon$ -amino group of D-Lysine. We had previously found that modified D-Lysine amino acids yielded satisfactory incorporation levels. The strained alkene within norbornene is expected to display increased reactivity compared to the allyl group and has been previously used in live cell imaging.<sup>34-36</sup> Surfaces of *S. aureus* cells remodeled with D-allylglycine resulted in a 3-fold increase in fluorescence compared to unmodified control cells (Fig. 2). The incubation of the same cells with D-Lys-NB-OH resulted in a major increase (~30-fold) in fluorescence relative to control cells. We performed a fluorescence competition assay we recently developed to quantitatively establish incorporation efficiency of all alkene-displaying D-amino acid variants acids (Fig. S1, ESI†). As expected, D-allylglycine was incorporated onto cell surfaces to a higher extent than D-Lys-NB-OH. Furthermore, we observed much slower reaction kinetics with tetrazine for the unstrained alkene compared to the norbornene-bearing amino acid variants (Fig. S2, ESI†). Evidently, between the two opposing effects (incorporation efficiency and elevated strain-energy) the strained alkene is a more important overall feature.

Next, we evaluated the labeling efficiency of D-diaminopropionic acid modified with norbornene (D-Dap-NB-OH). Satisfactorily, the smaller D-Dap-NB-OH variant led to improved labeling levels relative to D-Lys-NB-OH (Fig. 2). Cells remodeled with D-Dap-NB-OH led to ~45-fold increase in fluorescence relative to control cells. Finally, we set out to evaluate the possibility that we could further improve labeling by modifying the C-terminus carboxylic acid functional group into carboxamide. We and others had previously discovered that amidation of the C-terminus can increase loading and retention of the D-amino acid on the cell surface.<sup>37, 38</sup> Consistent with our previous findings, we also showed that the carboxamide D-Dap-NB-NH<sub>2</sub> led to higher incorporation levels compared to its carboxylic acid counterpart (Fig. S1, ESI†). Cells labeled with the carboxamide D-Dap-NB-NH<sub>2</sub> led to a signal increase of ~55-fold compared to control cells. Together, we demonstrate that through structural optimization we were successful in using tetrazine ligation to site-specifically label the surface *S. aureus* cells.

A major advantage of tetrazine is its compatibility with live cell ligation.<sup>30, 33, 39-42</sup> Next, we evaluated the tetrazine ligation reaction with live *S. aureus* cells metabolically remodeled with D-Dap-NB-NH<sub>2</sub> (Fig. 3a). A time course analysis of the labeling with Cy5-methyl tetrazine showed robust labeling as early as 30 minutes and the signal continued to increase over the next several hours. The fast reaction kinetics of this ligation can potentially complement existing biocompatible reaction strategies. Fluorescence microscopy analysis of cells labeled with D-Dap-NB-NH<sub>2</sub> showed delineated labeling at the septal region of the cells, consistent with the site of new peptidoglycan biosynthesis (Fig. 3, inset). In addition, we performed two experiments to establish the mode of surface remodeling with D-Dap-NB-NH<sub>2</sub>. First, we isolated the peptidoglycan from cells labeled with D-Dap-NB-NH<sub>2</sub>, analysed by mass spectrometry, and identified fragments consistent with covalent incorporation into the peptidoglycan monomeric structure (Fig. S3, ESI†). Second, the same cells incubated with the enantiomer L-Dap-NB-NH<sub>2</sub> led to near base line fluorescence signals, a result that points to the requirement for the D-stereochemistry (Fig. S4, ESI†).

Ring strain of the alkene species has significant influence on reaction kinetics with the tetrazine ligation partner. The highly strained *trans*-cyclooctene (TCO) has been reported to accelerate the



**Fig. 3** Flow cytometry analysis of live *S. aureus* labeled with (a) D-Dap-NB-NH<sub>2</sub> or (b) D-Dap-Tet-NH<sub>2</sub>. Data are represented as mean + SD (n=3). *Inset top*, fluorescence microscopy imaging of *S. aureus*; scale bar is 2  $\mu$ m. *Inset bottom*, chemical structure of D-Dap-Tet-NH<sub>2</sub>.

reaction between 2 to 3 orders of magnitude.<sup>33</sup> We reasoned that the installation of TCO onto the sidechain of a D-amino acid would lead to minimal incorporation due to its large size. Instead, we explored the possibility of switching the ligation partners. We designed and synthesized D-Dap-Tet-NH<sub>2</sub>, in which the tetrazine was conjugated to the amino sidechain of D-Dap. In this scheme, the tetrazine is metabolically loaded onto the bacterial surface and TCO-conjugates can be used to further decorate the bacterial surface. *S. aureus* cells surfaces were remodeled with D-Dap-Tet-NH<sub>2</sub>, followed by the incubation with TCO-Cy5, and analyzed by flow cytometry. Specific labeling in the presence of the tetrazine-displaying D-amino acid was observed (Fig. 3b). However, the reaction was slower compared to D-Dap-NB-NH<sub>2</sub>. Presumably, the incorporation levels may have been lower than desired or the amino group adjacent to the tetrazine may deactivate the diene. Finally, we showed that the unnatural D-amino acid displayed no significant reduction in cellular viability (Fig. S5, ESI†). Future re-designs will be evaluated to optimize the neighboring groups to the tetrazine to accelerate reaction rates, while preserving design features that increase incorporation efficiency.



## Conclusions

In conclusion, we established a new peptidoglycan labeling approach using alkene-tetrazine biorthogonal chemistry. We have shown that norbornene containing D-amino acids are viable coupling tools to link a tetrazine fluorophore on the surface of bacterial cells. This can provide an alternative method of installing molecules of interest to the exterior of the cell. Peptidoglycan labeling of live bacteria through this ligation approach can pave the way for future *in vivo* studies due to its non-toxic effects and proven biocompatibility. Tetrazine ligation will be considered our primary target for future development of our D-amino acid Recruitment Therapy (DART) strategy.

## Notes and references

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