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Bioorthogonal labelling of living bacteria using unnatural amino acids containing nitrones and a nitrone derivative of vancomycin

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Unnatural D-amino acids bearing endocyclic nitrones were developed for live-cell labelling of the bacterial peptidoglycan layer. Metabolic incorporation of D-Lys and D-Ala derivatives bearing different endocyclic nitrones was observed in E. coli, L. innocua, and L. lactis. The incorporated nitrones of these bacteria then rapidly underwent strain-promoted alkyne-nitrone cycloaddition (SPANC) reactions affording chemically modified bacteria.

Incorporating unnatural chemical functionality in to the biomolecules of living systems has allowed for the labelling, tracking and deciphering of molecular mechanisms in complex environments. Bioorthogonal chemistry can then afford useful connections to accomplish these goals. For example, the Staudinger ligation is most well-known for being applied to the metabolic labelling of cell-surface glycans through the design of azide-derivatized sugars capable of cellular uptake and expression on cell surfaces. Azides and alkynes have since been incorporated in to viral capsids, peptides and nucleotides, and unnatural amino acids and sterols and unnatural amino acids for copper-catalyzed or strain-promoted cycloadditions with great success. Developing methods to incorporate functional bioorthogonal groups in biologically relevant substrates is therefore crucial to applying any chemistry as a labelling strategy.

Strain-promoted alkyne-nitrone cycloaddition (SPANC) reactions have been applied to biological labelling experiments by modifying proteins to bear nitrone functional groups. Acyclic nitrones have been introduced into proteins through oxidation of an N-terminal serine residue by sodium periodate to produce an aldehyde, followed by condensation with a hydroxylamine, resulting in a nitrone-tagged polypeptide. Subsequent SPANC reactions have allowed the fluorescent labelling of proteins in solution with the sole requirement of an N-terminal serine residue.

Human epidermal growth factor (EGF) has also been modified to bear an endocyclic nitrone through an NHS-mediated coupling reaction, allowing for fluorescent SPANC labelling of mammalian cell surfaces expressing EGF receptors. Thus, SPANC reactions are predisposed to applications in metabolic labelling. Recently, an endocyclic nitrone-modified variant of 3-deoxy-D-manno-oct-2-ulosonic acid (KDO) has been shown to be readily incorporated in E. coli cell surface lipopolysaccharides (LPS) by highly selective biosynthetic enzymes. This incorporation and subsequent labelling by a modified Kinugasa reaction marks the first metabolic incorporation of an endocyclic nitrone in bacteria.

Unnatural amino acids (UAAs) bearing bioorthogonal chemical groups have been incorporated into the proteins and peptidoglycans of bacteria through various approaches. Bacterial cell wall peptidoglycan (PG), which consists of glycan strands cross-linked by peptides containing D-amino acids, has been shown to accept incorporation of azide and alkyne-bearing unnatural D-amino acids. Furthermore, some steric bulk can be tolerated since fluorophore-tagged UAAs are incorporated as well. Incorporation of UAAs into bacterial PG appears to hinge primarily on the stereocchemistry of the amino acid. Since bacteria use D-amino acids to cross-link layers of PG to form a mesh-like sacculus surrounding the cell membrane and since D-amino acids are rarely employed in any other kingdom of life, this unique bacterial trait represents a substantial focal point of antibiotic research and PG dynamics have only recently begun to be studied with the help of UAAs. Herein we establish the first examples of stable endocyclic nitrone incorporation into the PG of different bacteria and demonstrate rapid labelling of bacteria through the use of nitrone-bearing UAAs.

To investigate the potential incorporation of endocyclic nitrones into the PG of bacterial cell walls, a series of nitrone-containing unnatural D-amino acids were prepared (Scheme 1). UAAs were designed to include a D-amino acid centre and the electron-donating nitrone (UAAs 1 and 3) while remaining as structurally similar to the parent D-amino acid as possible, whereas UAAs 2 and 4 were designed to include the electron-rich nitrone (for details of the synthesis see supporting information). With these UAAs analogues bearing nitrone functionality in hand, we next sought to...
investigate whether these UAA s would undergo metabolic incorporation into the PG.

Listeria innocua was chosen as a model organism for its non-infectious resemblance to the food-borne pathogen L. monocytogenes. L. innocua are gram-positive rod-shaped bacteria that thrive over a broad range of temperatures. To determine if UAA s 1 to 4 could be incorporated into L. innocua PG, bacteria were cultured for one hour in BH I media containing 5 mM of UAA, or equivalent volume of DMSO (vehicle control), before multiple washes in PBS and subsequent SPANC reaction on the living cells. All of the D-amino acids tested were able to effectively label the PG of L. innocua equally to or more efficiently than the azido D-alanine probe (Figure 1). Robust fluorescence signal was obtained for all samples, and is displayed above non-specific labelling of DIBO-Alexa488 treatment of cells cultured in the absence of UAA s. The greatest resolution and brightest fluorescent images were obtained using UAA 1. PG labelling was not observed in the absence of a ‘clickable’ amino acid, indicating that the fluorescence observed is the direct result of amino acid incorporation and subsequent SPANC. Once ubiquitous PG labelling with nitrone-modified UAA was achieved in L. innocua, the potential PG labelling of another gram-positive species, Lactococcus lactis, was evaluated using similar labelling conditions with the exception that the bacteria were shaken during the incubation period with the metabolic probe, since L. lactis lack flagella. Similar labelling was observed (see supporting information).

Nitrone-modified UAA s have been shown to be readily incorporated in the PG of gram-positive bacteria of contrasting morphologies and motility. Gram-negative bacteria pose additional challenges to PG labelling. UAA probes would be required to pass through the outer cell membrane before reaching the PG where potential incorporation would occur. The PG layer of gram-negative bacteria is also thinner than that of gram-positive bacteria, providing fewer opportunities for UAA incorporation in cross-linking peptides. For these reasons more rapid and efficient bioorthogonal reactions are desired for PG labelling in these species. To evaluate the ability of nitrone-modified UAA s to penetrate the cell membrane and label the PG of gram-negative bacteria, K12 Escherichia coli were grown in LB broth until exhibiting mid-log phase growth and were incubated with UAA s as described above, with comparison to azido D-alanine and employing DMSO as a negative control (see supporting information). The SPANC modification of PG in this case was observed, but is less prominent than the labelling observed in the PG labelling of gram-positive bacteria.

Once we had established SPANC as a visualization method for metabolically incorporated nitrone-bearing UAA probes for PG in gram-positive bacteria, we sought to explore alternate PG labelling methods that would expand the scope and potential applications of bioorthogonal SPANC. Recent literature has reported the use of vancomycin-functionalized magnetic nanoparticles as a non-covalent probe for capturing gram-positive bacteria. Vancomycin is a pentapeptide glycoprotein antibiotic natural product that targets the D-alanine-D-alanine motif in gram-positive PG.

Cell-surface fluorescent labelling of bacteria with vancomycin has not yet been reported using a two-stage ‘click’ chemistry method, although PG labelling with a fluorescent BODIPY modified vancomycin has been shown. SPANC modification of vancomycin allows the opportunity for modular labelling with other dyes and other bioorthogonal groups. The functionalization of vancomycin is simplified by the fact that there are two potential and mutually exclusive modification sites that are unlikely to disrupt the tertiary structure of the peptide. A primary amine located on the vancomycin sugar is ideal for standard coupling reactions with
activated carboxylic acid moieties, while only one carboxylic acid is exposed, as the rest of the amino acid functional groups exist as peptide bonds. Modification of vancomycin at the free carboxylic acid position causes the least perturbation of the antibiotic function. We therefore sought to functionalize vancomycin with an endocyclic nitrone at the free carboxylic acid residue, using a polyethylene glycol (PEG) spacer to increase the accessibility of the nitrone to a strained alkyne reacting partner. Nitrone 5 was reacted with 4,7,10-trioxa-1,13-tridecanediamine (diamine-PEG) in an HATU-mediated amide coupling reaction using the diamine-PEG linker in large excess to avoid coupling two equivalents of nitrone 5 to each PEG unit. The PEG-modified nitrone was then coupled to vancomycin hydrochloride in a similar fashion (Scheme 2). L. lactis were then cultured as described above, incubated with varying concentrations of 7 for 30 minutes, reacted with DIBO-Alexa488 in a SPANC reaction, and visualized by fluorescence microscopy. Cell surface labelling was observed with concentrations as low as 20 μM of 7, which displayed similar labelling efficiency at higher concentrations (Figure 2).

In summary, we have shown that four different UAs bearing endocyclic nitrone groups are stable and readily incorporated into the PG of gram-positive and gram-negative bacteria. This is the first example of nitrone functional groups being incorporated into peptide structures through metabolic labelling strategies. The nitrone group is robust and rapidly reacts via SPANC chemistry, which gives rise to efficient labelling of the PG with fluorescence moieties. Additionally, vancomycin was derivatized with an endocyclic nitrone and SPANC chemistry was then demonstrated to be able to further functionalize vancomycin in situ. These studies establish nitrone as a useful bioorthogonal group for studying different bacterial species.

Notes and references