ChemComm

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FEATURE ARTICLE

Glycans in pathogenic bacteria – potential for targeted covalent therapeutics and imaging agents

Cite this: DOI: 10.1039/x0xx00000x

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Received 00th January 2014,
Accepted 00th xxx 2014

DOI: 10.1039/x0xx00000x

www.rsc.org/

A substantial obstacle to the existing treatment of bacterial diseases is the lack of specific probes that can be used to diagnose and treat pathogenic bacteria in a selective manner while leaving the microbiome largely intact. To tackle this problem, there is an urgent need to develop pathogen-specific therapeutics and diagnostics. Here, we describe recent evidence that indicates distinctive glycans found exclusively on pathogenic bacteria could form the basis of targeted therapeutic and diagnostic strategies. In particular, we highlight the use of metabolic oligosaccharide engineering to covalently deliver therapeutics and imaging agents to bacterial glycans.

1. Introduction

The introduction of small molecule antibiotics revolutionized medicine. Prior to the discovery of antibiotics like penicillin, bacterial diseases ravaged mankind. Since that time, antibiotics have saved millions of lives. However, despite the undeniable impact antibiotics have had on curing bacterial diseases, existing antibiotics suffer from a number of drawbacks that must be overcome.

One widespread challenge in the health care industry is the emergence and spread of pathogenic bacterial strains that are resistant to existing antibiotics. In hospitals, 50% of *Staphylococcus aureus* isolates are methicillin resistant[1], and vancomycin, a drug once known as the "antibiotic of last resort," is no longer effective for some infected individuals.[2] To address this challenge, chemists have employed three main approaches. One common method focuses on altering first-generation antibiotics to create variants that circumvent antibiotic resistance mechanisms.[3] Another successful tactic emphasizes the discovery or development of novel classes of antibiotics.[3] The third course of action employs combination therapies that inactivate resistance mechanisms and thus restore the efficacy of the initial antibiotic.[4] However, the presence of sustained selective pressure combined with the mutability of bacteria produces drug resistant strains that continue to challenge chemists. The dwindling antibiotic pipeline has further eroded our ability to combat infection.[5]

Another challenge is the crucial role that microorganisms play in human health, and the unintended consequences antibiotics can have on our beneficial flora.[6] In the human body, microbial cells outnumber human cells by a factor of ten.[7] Disrupting the microbiome with a course of broad-spectrum antibiotics can alter the composition of gut bacteria for years, resulting in deleterious consequences on human health.[6] Indeed, patients suffering from stomach ulcers who were treated with antibiotics for one week had a shift in their gut microbiome that lasted for up to four years post-antibiotic treatment.[8] Such disturbances to the normal gut microbiota have been associated with obesity, autoimmune disorders, allergies, and malnutrition.[9, 10] Moreover, interference of the microbiota with oral antibiotics enables pathogens to gain a foothold in the gut.[11] Given the vital role of beneficial flora in human health, we need to establish new narrow spectrum therapeutics that do not disturb symbiotic bacteria.

The use of microorganism-specific antibiotics rather than broad-spectrum antibiotics slows the evolution and spread of antibiotic resistance[12] by minimizing the likelihood of resistance gene transfer across bacterial species. In addition, microorganism-specific antibiotics treat pathogenic bacteria in a discriminating manner while leaving the host microbiome largely intact. Therefore, this method mitigates immediate health problems and minimizes long-term deleterious effects on beneficial bacteria. The practical deployment of microorganism-specific therapeutics requires both narrow spectrum antibiotics and rapid diagnostic tests that pinpoint the organism responsible for a patient's infection. Therefore, there is an urgent need to develop novel antibiotics and diagnostics aimed at specific bacterial populations.

Bacterial glycans represent intriguing targets of therapeutics and diagnostics. They are linked to pathogenesis, have distinctive structures, and, in some cases, are present on only a small number of pathogenic bacteria.[13] Here we provide an overview of bacterial glycans and how they can be harnessed to diagnose and treat bacterial diseases in a discriminating manner. We begin with a brief overview of bacterial glycan structures and their links to pathogenesis. We then highlight approaches to metabolically label these glycans with chemical reporters. Finally, we describe approaches to covalently target bacterial glycans with therapeutics or imaging agents.

1.1 Bacterial glycans are attractive pathogen-specific targets

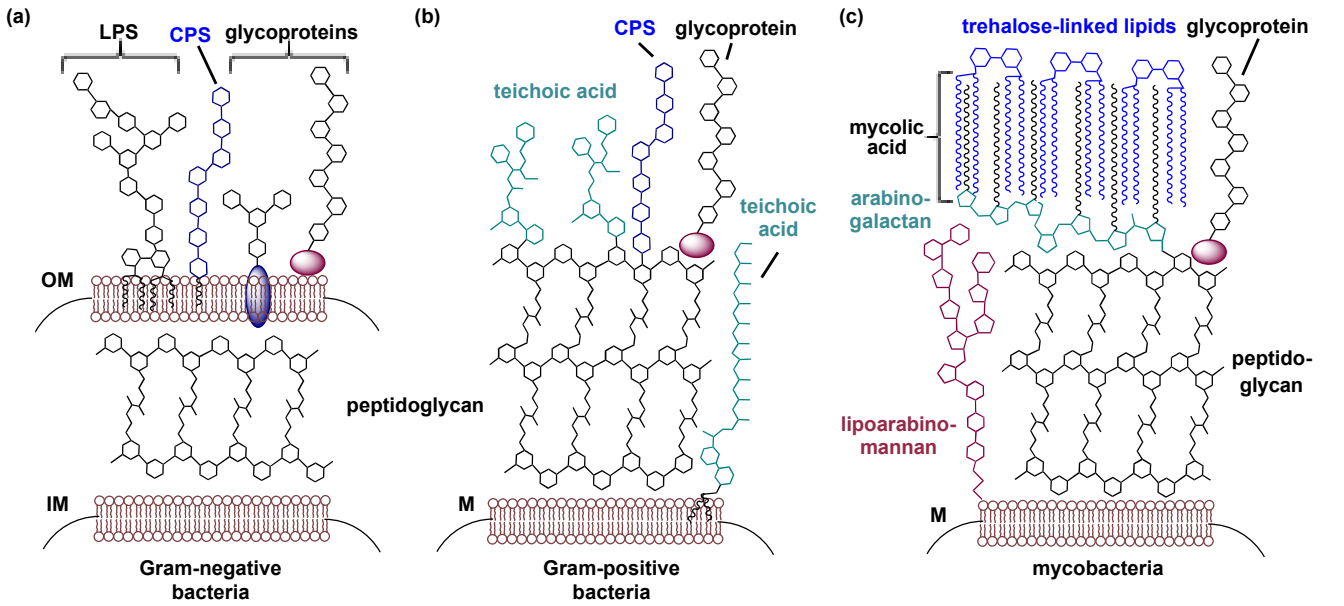

Fig. 1 Bacterial cell walls are coated with a diverse array of glycan structures. (a) Gram-negative bacteria contain both an inner membrane (IM) and an outer membrane (OM). The space between the membranes is rigidified by the peptidoglycan. Embedded in the outer membrane are lipopolysaccharide (LPS), capsular polysaccharide (CPS), and glycoproteins. (b) Gram-positive bacteria contain only one membrane (M), which is reinforced with a thick coating of peptidoglycan. Capsular polysaccharides, teichoic acids, and glycoproteins are found on the periphery of the cell. (c) Mycobacteria are Gram-positive bacteria that contain distinctive glycans on their cells, including lipoarabinomannan, arabinogalactan, mycolic acid, and trehalose-linked lipids. Image is not to scale.

Bacterial cells are coated with an impressive array of glycan structures that comprise their cell wall. The cell wall forms a suit of armour that protect the cell from its environment and osmotic lysis. Due to its critical importance in bacterial survival and its surface accessibility, the cell wall is a common target of antibiotics.[14] Blockbuster antibiotics such as penicillin[15], vancomycin[16], and bacitracin[17] all interfere with bacterial cell wall biosynthesis, which testifies to the cell wall's attractiveness as a drug target for novel therapeutics. The bacterial cell wall remains an exciting target, as it is covered with distinctive surface accessible structures that are linked to pathogenesis.

Most bacteria can be grouped into one of three categories depending on their cell wall architecture: Gram-negative bacteria, Gram-positive bacteria, and mycobacteria (Fig. 1).[18] Gram-negative bacteria have inner and outer cell membranes, with peptidoglycan in the intervening periplasmic space and lipopolysaccharide (LPS) and capsular polysaccharide (CPS) associated with the outer membrane (Fig. 1a).[19] Like Gram-negative cells, Gram-positive cells have a dense peptidoglycan layer and capsular polysaccharide on their cell surface (Fig. 1b). In contrast, Gram-positive bacteria contain only one cell membrane and produce teichoic acids on their surfaces rather than LPS.[19] Teichoic acids are either covalently attached to the peptidoglycan or anchored in the cell membrane via a lipid tail (Fig. 1b).[20] Finally, mycobacterial species, including the widespread human pathogen *Mycobacterium tuberculosis*, have such idiosyncratic cell walls that, although technically Gram-positive bacteria, they can be placed in a category of their own (Fig. 1c). The mycobacterial cell membrane is encased by a thick layer of peptidoglycan and lipoarabinomannan, which are further elaborated with arabinogalactan and mycolic acids, and finally capped with trehalose-linked lipids.[21] This exceptional cell wall has permeability characteristics that enable mycobacteria to evade antibiotics that target cell wall biosynthesis in Gram-negative and Gram-positive bacteria.[22] An additional differentiating feature is that some but not all strains of Gram-positive, Gram-negative, and mycobacteria also synthesize glycosylated proteins and present these glycoproteins on their cell surfaces.[13, 23] Regardless of bacterial sub-class, bacterial cells are covered with unusual glycans that are absent from human cells and have the potential to underpin novel therapeutic and diagnostic strategies.

An analysis of the structures of glycans found on bacterial cells reveals exclusively bacterial monosaccharide building blocks (Fig. 2). Some of these monosaccharides are widely prevalent in bacteria, while others are limited to a small number of bacterial pathogens.[13] For example, all bacterial cells are coated with peptidoglycan, a network of repeating units of β-1,4-linked *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic acid (MurNAc) crosslinked by short peptides (Fig. 2). Though GlcNAc is used broadly in prokaryotic and eukaryotic cells, MurNAc is a uniquely bacterial glycan. Thus, a therapeutic or imaging agent that targets MurNAc or peptidoglycan would influence all bacteria. In contrast, only Gram-negative bacteria synthesize LPS, which contains the distinctive monosaccharides 3-deoxy-D-manno-oct-2-ulosonic acid (Kdo) and L-glycero-D-mannoheptose (heptose; Fig. 2).[24] Consequently, an antibiotic or diagnostic that targets these structures would only impact Gram-negative bacteria. Similarly, Gram-positive cells are characterized by the presence of teichoic acids.[20] Hence, interfering with teichoic acid biosynthesis would impact only Gram-positive cells. Mycobacteria, in contrast, could be detected or targeted by their trehalose-linked lipids or arabinogalactan, for example (Fig. 2). These structures could form the basis of a strategy to diagnose or destroy each of the major classes of bacteria in a broad-spectrum manner.

Potentially attractive targets are rare carbohydrates, which are present on select bacteria and thus could form the

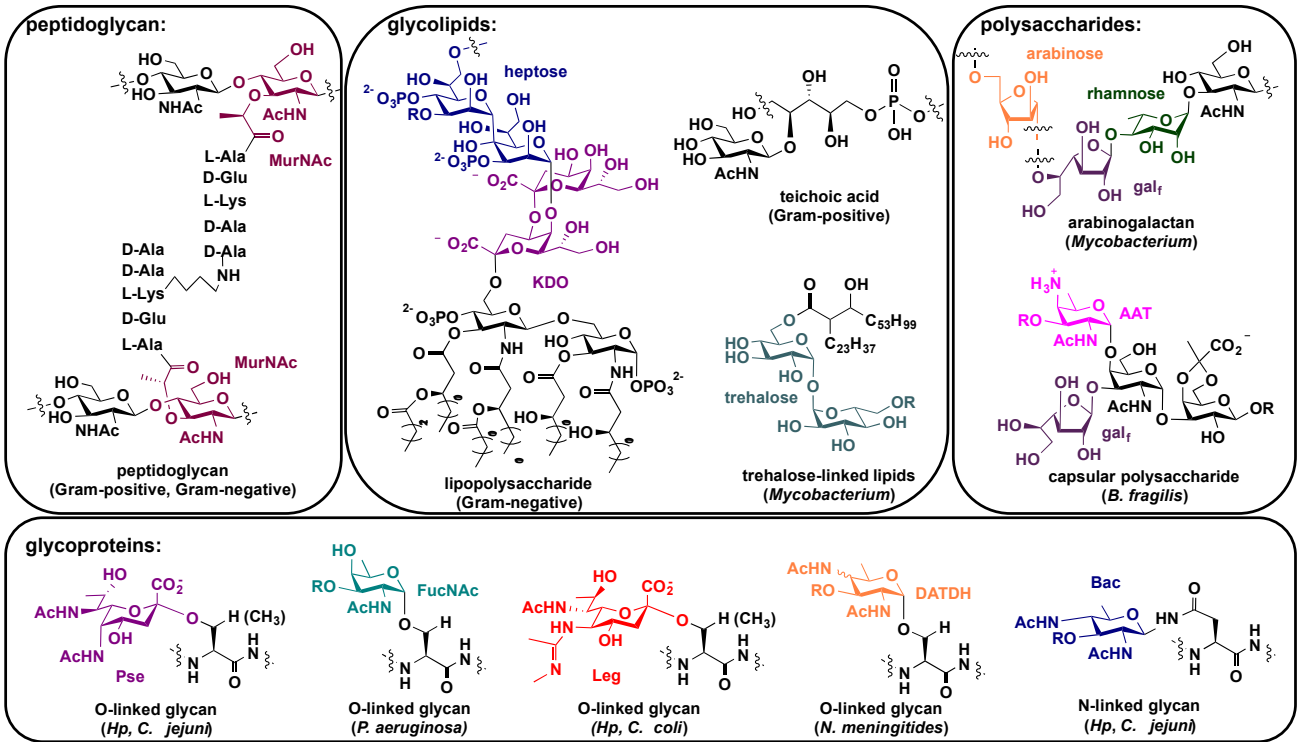

Fig. 2 Structures of representative glycans found on bacterial cells, with exclusively bacterial monosaccharides highlighted in color. Structures are grouped according to type of glycoconjugate, including peptidoglycan, glycolipids, polysaccharides, and glycoproteins, with known distribution indicated in parentheses. Abbreviations: MurNAc = muramic acid; KDO = 3-deoxy-D-manno-oct-2-ulosonic acid; heptose = L-glycero-D-mannoheptose; gal_f = galactofuranose; AAT = 2-acetamido-4-amino-2,4,6-trideoxyhexose; Pse = pseudaminic acid; Leg = legionaminic acid; DATDH = 2,4-diacetamido-2,4,6-trideoxyhexose; FucNAc = *N*-acetylfucosamine; Bac = bacillosamine

foundation of narrow-spectrum therapeutics and diagnostics. For example, *Neisseria meningitides* utilizes 2,4-diacetamido-2,4,6-trideoxyhexose (DATDH)[25], *Pseudomonas aeruginosa* installs FucNAc residues[26], and *Bacteroides fragilis* appends 2-acetamido-4-amino-2,4,6-trideoxy-galactose (AAT)[27] into its cell surface polysaccharides (Fig. 2). These distinctive building blocks are exclusively present on a small number of bacterial species.[13] Similarly, *Campylobacter jejuni*'s and *Helicobacter pylori*'s glycoproteins contain the amino- and deoxy-monosaccharides pseudaminic acid, legionaminic acid, and bacillosamine[28, 29] (see Fig. 2). These sugars have limited expression on pathogenic bacteria,[28, 30, 31] and there are no reports of these monosaccharides in commensal bacterial that dominate the human gut microbiome[9] (e.g. *Bacteroides sp.*, *Prevotella sp.*). These insights indicate that certain bacterial sugars are not widely used and are thus attractive targets of selective interference and examination.

Bacterial glycans are great targets because they are often linked to bacterial fitness and pathogenesis. Indeed, bacterial cells with improperly formed peptidoglycan undergo osmotic lysis[32], those with altered LPS are destroyed by the host's immune system[32], and bacteria that lack teichoic acids are attenuated in host colonization and infection.[33] Similarly, trehalose-deficient mycobacteria are not viable[34] and bacterial strains with inactivated protein glycosylation genes exhibit reduced host cell binding and colonization defects[28, 35-37]. These observations reveal that bacterial glycans perform crucial functions and are, in several cases, linked to pathogenicity. The ability to target these structures and visualize them could address the pressing needs to develop new antibiotics and diagnostics for bacterial diseases.

1.2 Traditional approaches to targeting bacterial glycans are powerful yet have limitations

Traditional approaches to targeting bacterial glycans for prophylactic or therapeutic purposes have had remarkable successes in the clinic.[13] For example, vaccines based on bacterial carbohydrate epitopes are at the forefront of preventative medicine and are used to immunize children against bacterial pathogens such as *Neisseria meningitides* (e.g. Menactra)[38], *Streptococcus pneumoniae* (e.g. Prevnar)[39], and *Haemophilus influenza* (Act-HIB)[40]. Continued efforts to develop a more extensive collection of anti-bacterial vaccines are underway. Though vaccination is a successful preventative measure, it does not help individuals who are already suffering from a bacterial infection. Therefore, antibiotics are critical for battling active infections. Our antibiotic arsenal contains numerous small molecule inhibitors of enzymes involved in bacterial glycoconjugate biosynthesis such as penicillin[15], vancomycin[16], and bacitracin[17] which interfere with peptidoglycan biosynthesis. Recent developments have enabled the elucidation of peptidoglycan biosynthesis[41], bacterial O-linked glycan biosynthesis[25], and rare monosaccharide biosynthetic pathways[42], thereby facilitating high-throughput screening of inhibitors of these pathways[42]. Despite their successes, traditional approaches that target bacteria by inhibiting cell wall assembly with therapeutics have limitations. Traditional antibiotics target molecules that promote growth.

As such, they miss a subset of bacteria that are not actively growing, in particular, dormant and persistent bacteria.[43] Additionally, some bacteria evade antibiotics due to their location within the host. Finally, the majority of antibiotics are broad-spectrum which endangers our beneficial flora. Thus, though traditional approaches to targeting bacterial glycans have much to offer to prevent and treat bacterial disease, complementary approaches should be explored.

In contrast, bacterial glycans have played a minimal role in diagnosing and tracking infection. The notable exception is with patient antisera, which often contains antibodies that bind to bacterial cell wall structures and can be used to establish whether a patient has had a particular bacterial infection.[44] Rather than using an indirect immune response in an *ex vivo* assay, efforts to image bacterial glycans in animal based models could lead to improved diagnostic capabilities.

New approaches that selectively target membrane glycans on both growing and persistent bacteria with therapeutics or imaging agents have the potential to address unmet needs. In this review, we focus on the potential benefits of covalent targeting of bacterial glycans with small molecule therapeutics and diagnostics. To ramp up the armamentarium against bacteria, we discuss a novel and highly modular approach to interfering with bacteria and imaging bacterial glycans based on metabolic labelling with bioorthogonal chemistries.

2. Metabolic labelling of bacterial glycans with chemical reporters

Metabolic oligosaccharide engineering (MOE)[45, 46] and bioorthogonal chemistry[47, 48] have the potential to transform the way bacterial diseases are treated and bacterial infections are visualized. MOE is a two-step chemical approach that was pioneered by Bertozzi[49, 50], Reutter[51, 52], and colleagues to study and target eukaryotic glycans and has been recently extended to bacterial glycans. In the first step of MOE, an unnatural sugar containing a bioorthogonal chemical reporter is taken up and processed by permissive carbohydrate biosynthetic enzymes in living cells, ultimately leading to the metabolic replacement of endogenous sugars with unnatural variants (Fig. 3). In the second step, cells that are covered with chemical reporters undergo a reaction with exquisitely selective reactive partners to yield covalent adducts (Fig. 3). Reactive partners can be conjugated to therapeutics or diagnostics to enable the covalent delivery of drugs for targeted bacterial killing or diagnostic imaging.

This versatile covalent targeting strategy leaves room for creativity and permits the design of a large variety of both broad- and narrow-spectrum therapeutics and diagnostics. Essentially, by treating a patient with a carefully chosen sugar pill followed by a therapeutic or diagnostic cocktail, bacterial cells could be targeted or visualized by virtue of their glycan coat. In this section we will discuss ways to make this strategy highly selective for certain populations of bacteria by modulating (1) the unnatural sugar substrate chosen for metabolic labelling and (2) the bioorthogonal chemistry used for glycan targeting. In the final section, we will discuss a plethora of options for covalent targeting of bacterial glycans with therapeutics or imaging agents.

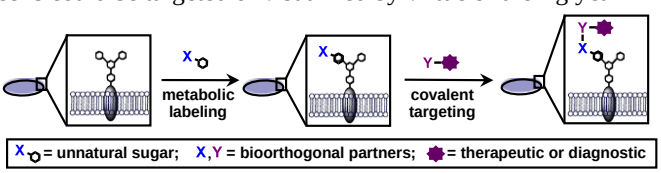

Figure 3. Metabolic oligosaccharide engineering can be employed to covalently deliver therapeutics or diagnostics to bacterial glycans. First, bacterial cells metabolically process an unnatural sugar that contains a bioorthogonal chemical reporter (X) into cellular glycans. In a second step, the chemical reporter undergoes covalent elaboration with a reactive partner (Y) conjugated to a therapeutic or diagnostic to form a covalent adduct.

2.1 Unnatural substrates for metabolic labelling of bacterial glycans

The first step of MOE requires an unnatural sugar that is taken up by bacteria and processed by the cell's metabolic pathways.[45, 46] Uptake is aided by transporters in the bacterial membrane[53] or by passive diffusion across the lipid bilayer, which can be facilitated by the temporary masking of hydrophilic hydroxyl groups with hydrophobic acetyl groups.[54] Once the unnatural sugars enter cells, non-specific esterases remove temporary protecting groups to yield free hydroxyls.[55] Permissive carbohydrate biosynthetic enzymes then process the unnatural sugars and incorporate them into surface glycans in place of natural sugars. As detailed below, several classes of bacterial glycans have been labelled with ketone ($-COCH_3$), azide ($-N_3$), or alkyne (-CCH) chemical reporters to date (Fig. 4). These examples illustrate that subtle structural perturbations are well tolerated by a variety of carbohydrate biosynthetic enzymes in bacteria, setting the stage to choose an appropriate metabolic precursor to label and target select bacterial glycans.

The pioneering report of metabolic labelling of bacterial glycans with chemical reporters employed a ketone-modified glycopeptide analogue of a naturally occurring peptidoglycan precursor, uridine diphosphate (UDP)-MurNAc pentapeptide (Fig. 4a).[56] In this study, Sadamoto and coworkers metabolically introduced ketones into the peptidoglycan of Gram-negative (*Escherichia coli*) and Gram-positive bacteria (*Lactobacillus* species) by supplementing the bacteria with a derivative of the precursor bearing a ketone modification at the peptide side chain (Fig. 4a). The cell wall precursor is a substrate of bacterial but not human cells and therefore should enable selective labelling of bacteria with ketones. Using a hydrazide-based probe to target the ketone moiety on the cell walls, these authors were able to modulate levels of bacterial adhesion, a key property that is essential for colonization and persistence.[57] As an alternative to labelling peptidoglycan with a glycan-containing metabolic precursor, supplementing bacteria with azide- and alkyne-bearing D-amino acids results in labelling of newly synthesized peptidoglycan (Fig. 4b), yet no labelling of proteins or teichoic acids.[58, 59] Notably, labelling with these peptidoglycan precursors occurs in Gram-positive bacteria, Gram-negative bacteria, and mycobacteria and thus could form the basis of a broad-spectrum antibiotic or bacterial imaging strategy.

Though peptidoglycan-labelling studies have involved chemically modifying the peptide moiety in their chosen metabolic precursors, subsequent metabolic labelling experiments with bacteria have primarily modified monosaccharide substrates. For example, Dumont *et al.* synthesized an alkyne-containing variant of Kdo (Fig. 4c), a characteristic monosaccharide present in the inner core of LPS, and reported that alkyne-modified Kdo is incorporated into Gram-negative bacteria (e.g. *E. coli*, *Salmonella typhimurium*, *Legionella pneumophila*) but not Gram-positive bacteria (e.g. *S. aureus* and *Bacillus subtilis*).[60] This observed labelling pattern is consistent with the presence of LPS on Gram-negative but not Gram-positive bacterial cells. Hence, antibiotics or

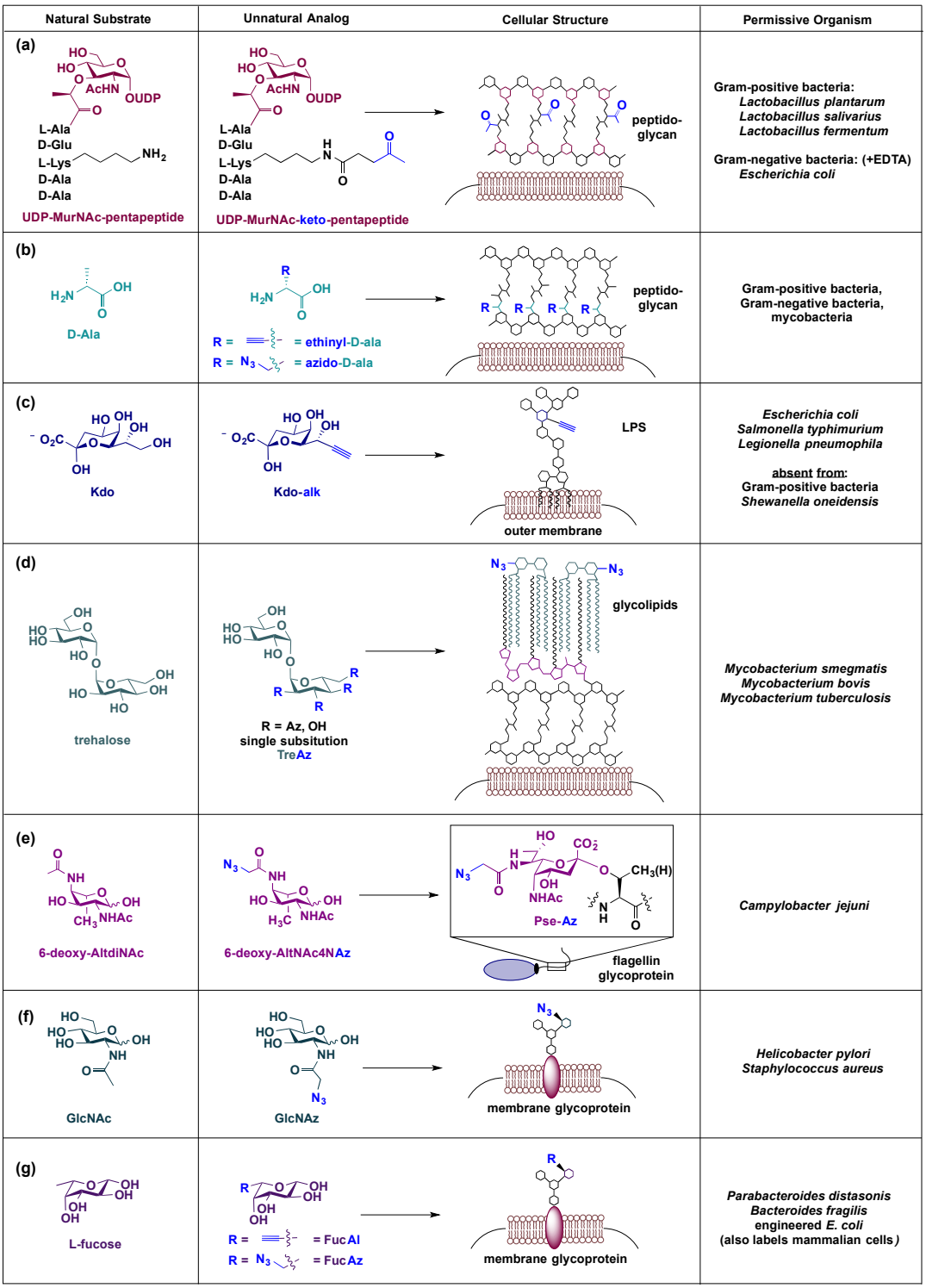

Fig. 4 Literature examples of unnatural metabolic precursors that are processed and incorporated into bacterial glycans. (a) Sadamoto *et al.* demonstrated that a ketone-bearing analog of the peptidoglycan precursor UDP-MurNAc-pentapeptide is metabolically incorporated into peptidoglycan on Gram-positive and Gram-negative bacteria.[56] (b) VanNieuwenhze, Bertozzi and colleagues found that azide- and alkyne-bearing analogs of D-amino acids are metabolically incorporated into peptidoglycan in myriad bacteria.[58,59] (c) Dumont *et al.* reported that an alkyne-containing variant of Kdo is incorporated into LPS of Gram-negative bacteria.[60] (d) Swarts *et al.* revealed that azide-containing trehalose derivatives are processed by Mycobacteria and incorporated into their glycolipids.[62] (e) Liu *et al.* found that 6-deoxy-AltNAc4NAz is processed by *C. jejuni*, converted to azido-pseudaminic acid, and displayed on flagellin glycoproteins.[63] (f) Champasa *et al.* established that GlcNAz is metabolically processed *into H. pylori's* glycoproteins.[64] Further, Memmel *et al.* found that GlcNAz is incorporated into *S. aureus'* surface glycans.[66] (g) Finally, Wu and coworkers demonstrated that both alkynyl and azide-containing fucose derivatives are tolerated by carbohydrate processing enzymes in *Parabacteroides sp.*[67] Abbreviations: MurNAc = muramic acid; D-Ala = D-alanine; Kdo = 3-deoxy-D-manno-oct-2-ulosonic acid; Pse = pseudaminic acid.

diagnostics that target alkyne-Kdo would only affect Gram-negative bacteria.

The development of alternative therapeutic approaches is particularly urgent for multidrug resistant bacteria such as *Mycobacterium tuberculosis*. Recent investigations into the unique outer membrane of mycobacteria reveals structures, such as trehalose monomycolate and trehalose dimycolate, that are essential for cell wall biosynthesis and disease progression.[61] By taking advantage of the natural trehalose recycling pathway, Bertozzi, Swarts, and coworkers selectively labelled glycolipids on *Mycobacterium smegmatis* cells with four distinct azido-trehalose (TreAz) derivatives (Fig. 4d).[62] TreAz containing glycolipids were successfully installed in the mycobacterium cell surfaces, which allowed for targeted delivery of azide-specific cyclooctyne probes.[62] Given the narrow expression of trehalose on mycobacteria and its absence from Gram-negative and Gram-positive bacteria, metabolic labelling with TreAz derivatives has the potential to yield selective labelling of mycobacterial cells with azides and therefore could form the basis of mycobacteria-specific therapeutics and diagnostics.

Narrow-spectrum therapeutics and diagnostics could be developed based on the expression of certain rare monosaccharides on bacteria. For example, pseudaminic acid is present on only a small number of pathogenic bacteria, while neither pseudaminic acid nor any enzyme in its biosynthetic pathway are found in mammalian cells.[28, 42] Intrigued by this knowledge, Liu *et al.* designed and synthesized an azide-containing variant of 6-deoxy-AltdiNAc, a dedicated precursor to pseudaminic acid.[63] The authors demonstrated that *C. jejuni*'s pseudaminic acid biosynthetic pathway is permissive of the azide-containing substrate 6-deoxy-AltNAc-4-NAz and that this substrate has only one fate in *C. jejuni* – conversion into azido-pseudaminic acid and incorporation into flagellin glycoproteins (Fig. 4e).[63] Thus, this work indicates that pseudaminic acid biosynthesis could be exploited to incorporate azides selectively onto a handful of pathogenic bacterial cells for therapeutic or diagnostic purposes.

The discovery and access to unique bacterial sugars for targeting specific bacterial populations remains the chief bottleneck in MOE. Current efforts to profile bacterial glycan structures could yield additional unique glycan structures to augment this targeting approach. Indeed, several rare hexosamine bacterial monosaccharides such as FucNAc, Bac, AAT, and DATDH have been identified. Synthetic methods developed by Kulkarni and colleagues have permitted unprecedented and expedited access to these rare sugars.[64, 65] Synthetic access to these monosaccharides opens the door to the creation of chemical reporter-containing variants of these sugars and thus expands the possibilities for metabolic labelling of unique bacterial glycans.[64, 65]

In addition to uniquely bacterial glycans, sugars common to both bacterial and mammalian cells can also be utilized in this approach. Selective targeting of bacteria with common sugars is possible if the sugars are *differentially* incorporated onto bacterial cells compared to mammalian cells. For example, GlcNAc is a common building block present in N-linked glycoproteins on the surface of mammalian cells. Surprisingly, metabolic labelling of *H. pylori* and mammalian cells with peracetylated *N*-azidoacetylglucosamine (GlcNAz) led to appreciable levels of azide-labelled glycans on the surface of *H. pylori*[66], yet not on the surface of kidney epithelial cells[67] (Fig. 4f). The selective incorporation of the common metabolic precursor GlcNAz on bacterial cell surfaces provides an intriguing area of pursuit for preferentially tagging bacteria in the context of mammalian cells. Recent work by Memmel *et al.* revealed that *S. aureus* also metabolically incorporate GlcNAz into surface glycans.[68] Intriguingly, azide-functionalized *S. aureus* covalently targeted with alkyne dyes display reduced adherence to host cells. These exciting studies indicate that even broad metabolic precursors could enable selective targeting of bacteria. GlcNAz may represent a special case, however, as azide and alkyne derivatives of the widespread sugar L-fucose are incorporated into fucosylated glycans on both bacterial (e.g. *Parabacteroides* sp. and engineered *E. coli*)[69] and mammalian cell's surfaces[70, 71] (Fig. 4g). Nevertheless, the GlcNAz results suggests that the selective labelling of bacteria with unnatural derivatives of common metabolic sugars merits investigation.

The above-described studies establish that distinctive glycans found in the cell wall of bacteria can be metabolically labelled with dedicated precursors containing chemical reporters. Labelled bacteria are poised for targeting with reactive partners for imaging or selective damage. The breadth of these examples underscores the impact of metabolic labelling for potential therapeutic and diagnostic applications. We are now poised to develop new unnatural sugars to specifically target a narrow range of bacteria using myriad biocompatible chemistries discussed in the next section.

2.2 Bioorthogonal chemistries for glycan targeting

Covalent targeting of bacterial glycans requires chemistries that are compatible with living systems. Ideally, the chemistries chosen should employ chemical reporters that are normally absent from biological systems, stable in water, nonreactive with biological functional groups, and capable of undergoing covalent elaboration with exquisitely selective reactive partners under physiological conditions.[48] Several chemistries have been developed that meet these demands; these "bioorthogonal" chemistries are discussed in depth in recent reviews.[72-74] In this section, we highlight select bioorthogonal chemistries that demonstrate the potential to covalently target bacterial glycans in living systems (Fig. 5). Crucially, these chemistries use small chemical reporters that are likely to be tolerated by permissive carbohydrate biosynthetic pathways and unlikely to perturb the function of labelled glycans. Moreover, the chemistries we discuss have the potential to proceed *in vivo* at appreciable rates, either on their own or with the aid of a biocompatible catalyst. We will briefly enumerate the application of these chemistries in the context of glycans and highlight their relative advantages and disadvantages in cell and animal based applications.

As discussed in the previous section, ketones, azides and alkynes are chemical reporters that have been used to label bacterial glycans. Originally, ketones were explored as chemical reporters because they are virtually absent from cell surfaces and are not found in naturally occurring amino acids, glycoconjugates, or lipids.[49] Ketones undergo covalent elaboration with hydrazide and amino-oxy probes in near neutral environments (pH 5-6) to form stable hydrazone or oxime products[48], respectively (Fig. 5a, b).[75, 76] Although the resulting oxime products are more stable to hydrolysis than hydrazones[77], ketone ligation with hydrazide proceeds more favourably at biological pH.[76] Remarkably, ketone-hydrazide chemistry is bioinert in the extracellular environment and has been previously accomplished in both Gram-negative and

Chemical Reporter	Reactive Partner	Covalent Product	Notes
ketone	(a) hydrazide; k = 0.033 $M^{-1}s^{-1}$	hydrazone	optimal at pH 5-6; proceeds on live bacterial and mammalian cells
	(b) amino-oxy; k = 0.057 $M^{-1}s^{-1}$	oxime	
azide	(c) Staudinger ligation: phosphine; k = 0.0038 $M^{-1}s^{-1}$	phosphine oxide	proceeds in mice without apparent detriment to physiology
	(d) Cu(I)-catalyzed azide-alkyne cycloaddition: alkyne; Cu(I); k = 1-10 $M^{-1}s^{-1}$	triazole	Cu(I) is biocompatible in the presence of H_2O-soluble ligands; proceeds in bacterial cells
	(e) Strain-promoted azide-alkyne cycloaddition: cyclooctyne; k = 10^{-3} - 10^{0} $M^{-1}s^{-1}$	triazole	proceeds in mice without apparent detriment to physiology
	(f) Photo-click azide alkyne cycloaddition: acetylene; 350 nm; k = 0.039 $M^{-1}s^{-1}$	triazole	light-initiated reaction; proceeds in cells
alkyne	(g) Cu(I)-catalyzed azide-alkyne cycloaddition: azide; Cu(I); k = 1-10 $M^{-1}s^{-1}$	triazole	Cu(I) is biocompatible in the presence of H_2O-soluble ligands; proceeds in bacterial cells
	(h) Pd(II) mediated Sonogashira coupling: iodophenol; $Pd(NO_3)_2$	pyrazoline	$Pd(NO_3)_2$ exhibits low toxicity; proceeds in Gram-negative cells; rates comparable to Cu(I) cycloaddition with BTAA ligand
cyclopropene, alkene	(i) Inverse electron demand diels Alder: tetrazine; k = 10^{-2} $M^{-1}s^{-1}$	dihydropyrazine	labels surface glycans on live mammalian cells
isomeric cyclopropene	(j) 1,3 dipolar cycloaddition: nitrile imine; UV	cycloadduct	labels proteins in PBS
alkene	(k) Photo-activatable 1,3 cycloaddition: tetrazole; 365 nm; k = 0.79 $M^{-1}s^{-1}$		ligation product is fluorescent, labels proteins in mammalian cells
isonitrile	(l) tetrazine; k = 0.12 $M^{-1}s^{-1}$		labels surface glycans on live mammalian cells
vinyl sulfide	(m) Hetero Diels Alder: o-quinone methide; k = 0.0015 $M^{-1}s^{-1}$	thioacetal	proceeds in mammalian cells

Fig. 5 Reported bioorthogonal chemistries with promise for use in covalent targeting of bacterial glycans. The highlighted chemistries entail small chemical reporters that are distinctive in biological systems, bioinert, and undergo exquisitely selective covalent elaboration with the indicated reactive partners. The second order rate constants were reported in the following references: (a)[75], (b)[76], (c)[83], (d)[86], (e)[73], (f)[98], (g)[86], (h)[110], (i)[113,116], (j)[114], (k)[115], (l)[118], (m)[119].

Gram-positive bacteria.[49, 78] Furthermore, Rideout and coworkers demonstrated that this chemistry is robust even in the context of a living animal.[79, 80] Thus, bacteria labelled with ketones are poised for safe and selective covalent targeting with therapeutics and imaging agents within a host.

The introduction of the azide as a reporter in biological systems represents a turning point in the development of bioorthogonal chemistries, as azides are truly absent from biological systems.[47] Unlike their inorganic counterparts, alkyl azides are relatively nontoxic and are sufficiently tolerated by cells[50] and whole organisms[81, 82]. Organic azides act as soft nucleophiles that react with select partners to form adducts. For example, azides react with triarylphosphines via Staudinger ligation to yield amides (Fig. 5c).[83] This robust chemistry proceeds in mice without side reactions or apparent detriment to physiology.[47] Therefore, the Staudinger ligation has properties that make it well suited to probe bacterial glycans in the context of infected individuals. Although the Staudinger ligation is highly selective, triarylphosphines are easily inactivated through oxidation and have slow reaction kinetics[47], which could pose pharmacokinetic liabilities for targeting bacterial glycans *in vivo*.

Therefore, alternative azide-based bioorthogonal reactions have been explored. Meldal, Sharpless and coworkers demonstrated that Cu(I)-catalysed azide-alkyne cycloaddition, also known as "click chemistry," is highly selective[84, 85] and incredibly fast ($k = 1\text{-}10\ M^{-1}s^{-1}$)[86] (Fig. 5d). However, the initial application of click chemistry *in vivo* was fairly limited due to the high toxicity profile of copper[87, 88] at the concentration required to enable catalysis.[84] To enhance the biocompatibility of this chemistry, Bertozzi and coworkers developed the "strain promoted azide-alkyne cycloaddition" (SPAAC), a bioorthogonal reaction between azides and strained cyclooctynes to yield triazoles (Fig. 5e).[89, 90] Alterations to the cyclooctyne scaffold have led to a 100-fold improvement in reaction rates relative to the Staudinger ligation.[73, 91] Indeed, the rates of reaction for the most recently developed reagents for SPAAC are comparable to that of Cu(I)-catalysed click chemistry.[73, 92] Further, SPAAC proceeds in living animals, including in zebrafish[93-95], nematodes[96], and mice[97]. The light activatable conversion of cyclopropenes to cyclooctynes enables temporal, light-dependent control over SPAAC (Fig. 5f).[98] Taken together, SPAAC offers an attractive choice for targeting bacterial glycans. This chemistry is not without problems as some cyclooctynes react promiscuously with endogenous nucleophiles, such as free thiols[99], in the serum and in intracellular spaces. Modifications to the cyclooctyne scaffold, however, have been able to fine-tune reactivity, minimizing side reactions while maximizing biocompatibility.[100, 101]

An alternative approach to applying click chemistry in live cell imaging has relied on the deployment of copper chelators that mitigate Cu(I) toxicity. In particular, Finn and others have developed a number of water-soluble ligands that stabilize copper, accelerate click chemistry, prevent the formation of undesirable side products and oxidative damage to biomolecules, and ultimately sequester copper ions to facilitate removal.[102] These ligands enable click chemistry to proceed in cells with minimal loss of cellular viability.[102-107] Building upon this approach, Ting, Zhu, and co-workers employed an exciting strategy in their ligand design, which uses an azide-containing copper chelating agent. Ultimately, azide-containing copper ligands increase the effective concentration of Cu(I) at the catalytic site while reducing the actual copper concentration to the low micromolar range, at which copper toxicity is minimal.[108, 109] The use of copper chelators has vastly enhanced the viability of the alkyne as a chemical reporter in multicellular organisms.

Like azides, terminal alkynes are rarely seen in biological chemical space[48] and are small enough to minimize functional and structural perturbations. As a chemical reporter, alkynes can react with exogenous azides via Cu(I)-catalysed click chemistry, as discussed above (Fig. 5g). Alternatively, alkynes can react with indophenols in the presence of catalytic $Pd(NO_3)_2$ via Sonogashira cross coupling (Fig. 5h).[110] This reaction proceeds at a rate comparable to that of ligand-mediated Cu(I)-catalysed click chemistry, exhibits excellent compatibility with proteins, and proceeds to completion in *E. coli*, *Shigella* and *Salmonella*.[110] Especially intriguing, palladium toxicity[111] appears lower than copper toxicity[112]. Taken together, the palladium-catalysed Sonogashira cross coupling offers a feasible option for probing bacterial glycans and holds several exciting possibilities for future developments.

In addition to the well-established ketone, azide, and alkyne chemical reporters, other functional groups have arisen as promising chemical reporters. In particular, Prescher and coworkers have demonstrated that the cyclopropene is tolerated by permissive carbohydrate biosynthetic pathways in mammalian cells and undergoes covalent modification with tetrazine conjugates on live cells (Fig. 5i).[113] This chemistry proceeds at a rate comparable to the Staudinger ligation and is compatible with SPAAC between azides and cyclooctynes.[113] Isomeric 3,3-disubstituted cyclopropenes also have the potential to serve as orthogonal chemical reporters, as they do not react with tetrazine but instead selectively react with nitrile imines to form a cycloadduct upon UV light activation (Fig. 5j).[114] Thus, cyclopropenes are a viable approach to probing multiple bacterial glycans in tandem.

Terminal alkenes have also been explored as chemical reporters. Although alkenes are abundant in membrane lipids, endogenous alkenes are not accessible to chemical reactions because they are tightly packed within the lipid bilayer.[115] Thus, exposed terminal alkenes on the cell surface represent a distinctive reactive group that can be preferentially tagged with bioorthogonal chemistries. Wittman *et al.* reported that the sialic acid biosynthetic pathway is permissive of an unnatural substrate bearing an alkene, allowing terminal alkenes to be incorporated into mammalian glycans.[116] Further, these authors demonstrated that alkene-covered cells undergo highly selective ligation with tetrazine via an inverse Diels Alder (Fig. 5i).[116] Moreover, this chemistry proceeds selectively in the presence of azides.[116] Though cyclizations of tetrazines with terminal alkenes are slower than with cyclopropenes due to the lack of ring strain, modulating the tetrazine scaffold achieves a rate of reaction similar to the Staudinger ligation[116]. Alternatively, terminal alkenes undergo reaction with diaryl tetrazole via a light-mediated reaction to produce nitrile imines in mammalian cells (Fig. 5k).[115] The resulting product is a highly fluorescent pyrazoline cycloadduct, and the reaction proceeds without detriment to living cells when activated by a two photon 700 nm laser.[115] Thus, this chemistry has the potential to enable tracking of glycans in real-time.[115]

Isonitriles also have demonstrated utility in live cells.[117] This functional group are net neutral, small, stable at neutral pH, and display no appreciable toxicity in mammals. Isonitriles undergo [4+1] cycloaddition with tetrazines at a rate comparable to second-generation strain promoted cyclooadditions (Fig. 5l).[117, 118] Metabolic labelling of mammalian glycans with isonitrile-

modified sugar substrates proceeds without cytotoxic or cytostatic effects.[117] Moreover, isonitrile-tetrazine chemistry proceeds on live cells and is orthogonal to azide/cyclooctyne chemistry.[117] Thus, isonitriles are yet another viable glycan reporter.

Finally, vinyl thioethers have emerged as chemical reporters. Vinyl thioethers are small, chemically stable, and act as electron-rich dienophiles in a hetero-Diels-Alder cycloaddition with o-quinone-methides (Fig. 5m).[119] The rate of this reaction is on par with the Staudinger ligation, and the reaction is selective in the presence of thiols.[119] Although this chemistry has not yet been performed to label and track glycans, Li *et al.* reported that this cycloaddition proceeds in live mammalian cells and could be used to localize exogenous vinyl thioether conjugated taxol in mammalian systems.[119] Thus, vinyl thioethers could underlie a chemical strategy to visualize and target bacterial glycans.

In sum, the recent explosion of bioorthogonal chemistries has expanded the repertoire of chemical reporters that could be used for probing bacterial glycans. Further, the diverse modes of reactivity exhibited by these chemical reporters sets the stage for simultaneously tracking multiple bacterial glycans in living systems. Progress in translating these new chemistries into disease models and applying them to study bacterial glycans will provide a foundation for enhancing our understanding of host-pathogen interactions and for increasing our antibiotic repertoire.

3. Covalent targeting of bacterial glycans with therapeutics or imaging agents

Once bacterial glycans are metabolically labelled with chemical reporters, they are poised for covalent elaboration with selective reactive partners. Reactive probes that are conjugated to a therapeutic agent could catalyse cellular damage. Alternatively, reactive partners that comprise an imaging agent could be used to monitor dynamic changes in the bacterial glycan coat and disease progression. Below, we discuss recent examples of covalent targeting of bacterial glycans with therapeutics or diagnostics and highlight possible future directions.

3.1 Therapeutics that catalyse cellular damage or render bacteria innocuous

Covalent targeting of distinctive bacterial glycans with therapeutics has the potential to incite selective cellular damage or render bacteria harmless. Only two examples of covalently targeting bacterial glycans with therapeutics have been published to date. In the first example, Kaewsapsak *et al.* were able to recruit immune effector cells to target *H. pylori's* surface glycans.[67] Briefly, azide-covered *H. pylori* cells were covalently targeted via Staudinger ligation with phosphines conjugated to the immune stimulant 2,4-dinitrophenol (DNP) (Fig. 6a).[67] DNP-modified *H. pylori* were then exposed to anti-DNP antibodies and immune effector cells for targeted *H. pylori* killing (Fig. 6a).[67] Given the occurrence of naturally circulating anti-DNP antibodies in a high percentage of the human population, this bacterial targeting strategy has the potential to proceed in animals.[120] Indeed, the level of *H. pylori* killing observed was on par with the requirements for anti-DNP mediated cell killing of cancer cells targeted with DNP-conjugates *in vivo*.[121] This work establishes an important precedent for using immune stimulants for targeting bacterial glycans and could be extended to targeting diverse bacterial glycans with assorted immune stimulants, such as fluorescein, alpha-Gal, or 1,3 diketones.[122]

Rather than catalysing cell killing, an alternative therapeutic approach involves rendering bacteria innocuous within a host. Memmel *et al.* published a beautiful demonstration of this aim, in which they interfered with bacterial adhesion to host cells.[68] In essence, they demonstrated that covalent delivery of a bulky fluorophore, alkyne-Alexa-488, to GlcNAz-covered *S. aureus* via Cu(I)-catalysed click chemistry led to reduced adhesion to host cells relative to controls (Fig. 6b).[68] Given the crucial role of bacterial adhesion in infection[123] and bacterial persistence[124] in a host, inhibiting this process is an effective mechanism for drug therapy. The use of a fluorescent group provides the added benefit for tracking the allocation of the therapeutic in patients. This strategy has the potential to be broadened to an array of therapeutics that interfere with bacterial glycan function.

Although there are only two precedented examples of covalent targeting of bacterial glycans with therapeutics, we can take cues from the area of cancer research for inspiration on alternative therapeutics to target bacterial glycans. Here we discuss novel classes of therapeutics such as photosensitizers[125] and nanoparticles[126], and the use of toxins[127] to catalyse damage to bacteria.

In photodynamic therapy, photosensitizers generate toxic reactive oxygen species when excited by light (Fig. 6c).[128] The generated species then oxidize essential biological molecules and cause cell death (Fig. 6c).[128] Since singlet oxygen species have an incredibly short life span in water, their toxicity is localized in the immediate vicinity where they are produced; thus, targeted cells are killed with selectivity.[129] Several porphyrin-based photosensitizers are clinically approved for photodynamic eradication of cancer cells in the skin, head, neck, esophagus, lungs, and stomach.[128] In addition, photodynamic therapy has been applied for the treatment of bacterial infection.[130-132] Therefore, photosensitizer-containing reactive partners hold great potential for covalently targeting

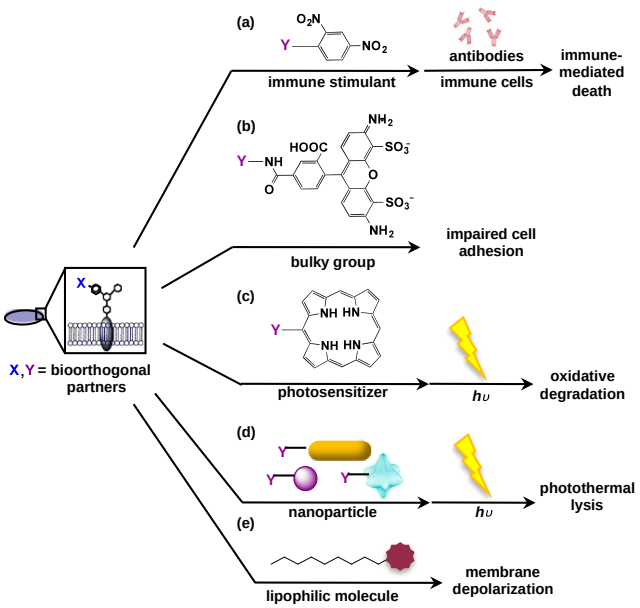

Fig. 6 Several classes of therapeutics, including (a) immune stimulants, (b) bulky groups, (c) photosensitizers, (d) nanoparticles, and (e) lipophilic molecules, have the potential to catalyse cellular damage or render bacteria innocuous if they are covalently delivered to chemical reporter-labelled glycans on bacterial cells.

bacteria based on their distinctive glycans.

Antibiotic development has the potential to benefit from the burgeoning field of nanomedicine. Although nanoparticles are in their infancy and are just entering clinical trials, they display unique optical properties that could be harnessed for imaging and therapeutic purposes. In particular, nanoparticles can be tuned to absorb light in the NIR region and subsequently dissipate heat (Fig. 6d).[133] Localized heating can disrupt cellular function or induce cellular lysis, ultimately leading to cellular destruction (Fig. 6d).[133] Gold nanorods remain one of most popular agents for photothermal therapy as gold is bioinert. However, other nanostructures have been identified as great photothermal agents.[133] One of the advantages of nanoparticles is that they can be tuned to absorb light at wavelengths that optimize depth of tissue penetration and targeted cell damage.[134] In addition, their surface chemistries can be modified to promote solubility and biocompatibility. The light dependent cytotoxicity of nanoparticles makes these particles optimal for antimicrobial therapeutics. Indeed, Norman *et al*. harnessed the power of antibody conjugated gold nanorods to trigger targeted photothermal lysis on Gram-negative *Pseudomonas aeruginosa*.[135] This study demonstrates that nanoparticles are feasible therapeutic agents for treating antibiotic resistant bacteria.

As an alternative to light-induced cell killing, lipophilic toxins induce cytoxicity via action on the bacterial membrane. Bacteria possess a particularly high transmembrane potential compared to human cells, and the maintenance of this potential is essential for proper cell function. Small lipophilic molecules can distort the cellular membrane and cause ion leakage that results in a change of proton motive force, causing cell death (Fig. 6e).[136] Lipophilic toxins, such as daptomycin, have demonstrated the ability to depolarize bacterial membranes and remain the last line of defence for treating drug resistant bacteria.[136] However, evidence of bacterial resistance to daptomycin is emerging. One hypothesized mechanism of resistance involves the loss of a docking site for daptomycin on the bacteria membrane.[136] The covalent attachment of daptomycin to bacterial cell wall via bioorthogonal chemistry could provide the antibiotic with an anchor to evade current mechanisms of resistance and restore biocidal activity. In addition, targeted delivery of membrane toxins to select bacteria would reduce the risk of nonspecific damage to human cells, potentially revitalizing the utility of lipophilic agents that have been dismissed in clinical trials due to unintended side effects. Therefore, targeting bacterial glycans with conjugated lipophilic agents is worthy of exploration.

In conclusion, the targeted delivery of therapeutics to bacterial glycans has the potential to induce specific cytotoxicity, thus minimizing harm to mammalian cells and beneficial flora. Although there are some direct precedents for targeting bacterial glycans with agents that induce cell death or render bacteria safe, there are significant prospects for growth in this area. Ultimately, the covalent delivery of therapeutics to bacterial glycans could expand new antibiotic strategies.

3.2 Imaging metabolically labelled bacterial glycans

Distinctive glycans that are exclusively present on bacterial cells have the potential to form the basis of molecular imaging strategies to monitor bacterial disease progression in real time. Indeed, the ability to track bacterial glycans in their native physiological setting – ideally in a non-invasive manner – could reveal insights into disease initiation and progression, as well as the effectiveness of particular therapeutics at curing bacterial infections. Further, non-invasive imaging of bacterial glycans *in vivo* could reveal how bacteria modulate their glycan coat over the course of infection.

Visualizing the bacterial glycan coat in cellular contexts and in animal infection models requires an imaging agent that reports on bacterial glycan structures. Historically, glycan-binding proteins such as lectins and antibodies have been utilized to monitor cellular glycans.[137] For example, Mahal and Hsu developed lectin-based microarrays that report on glycan composition of live *E. coli*.[138] Additionally, Dufrêne and colleagues were able to explore the spatial arrangement of wall teichoic acids in living *Lactobacillus plantarum* cells using fluorescent concanavalin A lectin probes.[139] Though lectins have enabled the study of bacterial glycans in cellular settings, they have limited applications in animal infection models due to their poor tissue penetrance.

Alternatively, fluorescent derivatives of cell wall-binding antibiotics have been used to visualize cell wall biosynthesis and disruption. For example, Pereira *et al*. visualized new peptidoglycan biosynthesis at division septa in *S. aureus* using a fluorescent analogue of vancomycin.[140] Similarly, Carlson and co-workers developed a fluorescent cephalosporin analogue that binds selectively to a subset of penicillin-binding proteins and reveals *in vivo* labelling and visualization of cell wall biosynthesis in *B. subtilis* and *S. pneumonia*.[141] While fluorescent antibiotics display far better tissue penetrance than lectins and thus have potential for visualizing bacterial glycans in living organisms, these fluorescent antibiotics perturb bacterial populations under study.

MOE-based approaches have emerged as a complementary means to visualize bacterial glycans. MOE overcomes many of the challenges associated with lectins, antibodies, and fluorescent antibiotics. Advantages include the high tissue permeability endowed by small molecules and the low probability of disrupting bacterial populations under study. In a pioneering example of utilizing MOE as the basis of glycan imaging in live bacteria, Sadamoto *et al.* visualized peptidoglycan synthesis in both Gram-positive and Gram-negative bacteria by tagging ketone-labelled peptidoglycan with fluorescein-hydrazide probes.[56] Since that initial report, several labs have employed MOE to image bacterial glycans. For example, Dumont *et al.* visualized LPS biosynthesis in live bacteria using azide-Kdo and Cu(I)-catalysed click chemistry with a fluorescent alkyne and a biocompatible copper-chelator; these authors demonstrated that only Gram-negative bacteria were visualized using this approach.[60] Additionally, GlcNAz-labelled glycans have been imaged on the surface of *H. pylori*[67] and *S. aureus*[68] cells. Each of these examples demonstrates the utility of MOE as a strategy to image bacterial glycans.

Recent work has enabled imaging of trehalose analogues on mycobacteria. Backus *et al.* demonstrated that a fluorescein-containing trehalose analogue is incorporated into growing *M. tuberculosis*, even in the context of a macrophage infection model.[142] Thus, this fluorescent glycan visualizes *M. tuberculosis* infection and has the potential to serve as a possible diagnostic tool to label *M. tuberculosis* in an infected host. In another report, Swarts *et al.* found that mycobacterial trehalose biosynthetic pathways are widely tolerant of azide modifications on substrates and can be imaged with fluorescent cyclooctyne-based reporters.[62]

The use of a bioorthogonal chemical reporter is particularly advantageous over other labelling strategies, as it provides temporal control of probe delivery and offers the possibility to perform pulse-chase experiments. Indeed, VanNieuwenhze and

co-workers imaged newly synthesized peptidoglycan in an array of bacterial species in a pulse-chase fashion using a combination of fluorescent and chemical reporter-bearing D-amino acids.[58] These authors performed sequential peptidoglycan labelling and executed time-lapse microscopy to visualize real-time peptidoglycan synthesis. Bertozzi and co-workers built upon this work by incorporating alkyne-bearing D-amino acids into *Listeria monocytogenes* and visualizing peptidoglycan synthesis in real-time during macrophage infection.[59] These examples highlight the use of metabolic labelling as a facile, modular approach to probing bacterial glycan dynamics in a spatial and temporal manner.

The next big steps in this area will likely involve moving into animal infection models. Though bacteria have been imaged in mice previously (e.g. magnetic resonance imaging of iron-oxide nanoparticle-coated bacteria[143]; fluorescence imaging of labelled bacteria[144]), they have not been visualized in animals in a glycan-specific way. There is, however, significant potential for translating the tracking of bacterial glycans into animal infection models. Pulse-chase, time-lapse imaging of glycans using MOE with azide-cyclooctyne chemistry has been performed in live zebrafish[95]. Moreover, fluorescence and radionuclide imaging of labelled cellular glycans on cancer cells based on Staudinger ligation and

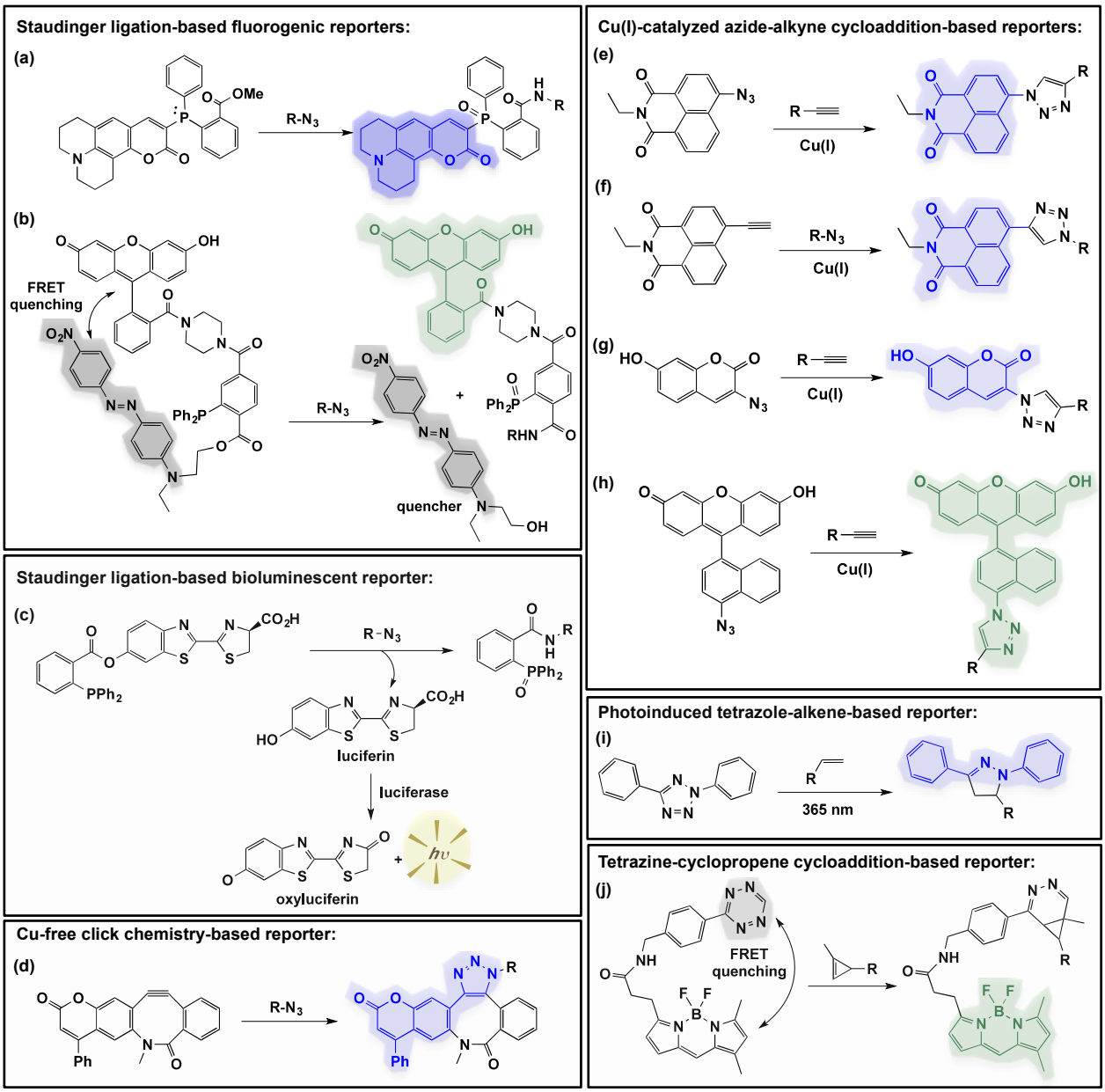

Fig. 7 Probes that are activated by bioorthogonal chemistries in cell-based settings. (a, b) Staudinger ligation-based fluorogenic reporters are activated by reaction with azides to yield fluorescent products. The probe in (a) is initially quenched by the phosphine lone pair electrons[147], whereas the probe in (b) utilizes a FRET quencher that is released upon Staudinger ligation[83]. (c) Staudinger ligation-based bioluminescent reporter that yields luciferin upon reaction with azides.[148] Once luciferin is released, luciferase-expressing cells catalyse the conversion of luciferin to ocyluciferin in a bioluminescent reaction that yields visible light. (d) Cu-free click chemistry-based reporter becomes fluorescent upon reaction with azides.[149] (e-h) Several Cu(I)-catalysed azide alkyne cycloaddition-based reporters become fluorescent when the azide or alkyne-based probe reacts with alkynes or azides, respectively.[70, 150, 151] (i) Photoinduced tetrazole-based reporter fluoresces blue upon reaction with alkenes.[115] (j) Tetrazine quenches BODIPY fluorescence; the conjugate becomes fluorescent when the tetrazine reacts with cyclopropene to yield a cycloadduct.[152]

SPAAC have been performed successfully in mice.[145, 146] Thus, literature precedents suggest that visualizing bacterial glycans and monitoring bacterial infection in animal models is just around the corner.

Non-invasive imaging of bacteria in animal models will be greatly aided by the development of "smart" probes that are activated upon reaction with bioorthogonal partners. Such "smart" probes enhance the signal to noise ratio while eliminating time required for probe removal. Fortunately, fluorogenic dyes that are activated by Staudinger ligation, Cu(I)-catalysed click chemistry, or SPAAC with azides have been introduced (Fig. 7). For example, Bertozzi and coworkers developed phosphine probes that become fluorescent or bioluminescent after reacting with azides (Fig. 7a-c).[83, 147, 148] Moreover, Jewett and Bertozzi developed a fluorogenic cyclooctyne that is activated upon reaction with azides (Fig. 7d).[149] Analogously, Wong, Bertozzi, and others introduced fluorogenic dyes that are activated by Cu(I)-catalysed click chemistry (Fig. 7e-h).[70, 150, 151] Future steps will likely focus on developing "smart" probes that extend beyond azide-chemical space and visualize some of the newer chemical reporters. Indeed, fluorogenic probes based on photo-click chemistry between tetrazoles and alkenes (Fig. 7i)[115] and between tetrazine-cyclopropene cycloaddition (Fig. 7j)[152] have been published and are important steps toward this goal. The development of probes activated by a variety of chemical reporters opens the door to tracking multiple bacterial glycans *in vivo* in parallel, thus enabling a thorough analysis of the bacterial glycan coat.

In sum, a number of exciting uses of MOE to image glycans on live bacterial cells have emerged. These examples set the stage for imaging bacterial glycans in normal and pathological settings, both in live bacteria and in animal models. Ultimately, this approach has the potential to enter the clinic for diagnosing disease and monitoring disease progression.

4. Conclusions

There are substantial challenges associated with existing antibiotics, including widespread antibiotic resistance and the unintended consequences of broad-spectrum antibiotics on our beneficial flora. To overcome these challenges, novel antibiotics and diagnostics aimed at specific bacterial populations are needed. Bacterial glycans are attractive targets of therapeutics and diagnostics because they are linked to pathogenesis, have distinctive structures, and are present on only a small number of pathogenic bacteria. MOE offers an innovative approach to modify bacterial glycans with chemical reporters in either a narrow-spectrum or broad-spectrum manner, depending on the choice of metabolic substrate. This highly modular approach can be tailored to applications of interest by modifying the unnatural substrate, the chemical reporter, the bioorthogonal chemistry, and the choice of therapeutic or imaging agent. Therefore, recent advances in MOE and bioorthogonal chemistry have laid the foundation for labelling distinctive bacterial glycan structures with chemical reporters, developing covalent therapeutics that target labelled glycans, and performing multiplex bacterial glycan imaging to monitor disease progression in real time.

Before MOE could be applied in a clinical context, either for therapeutics or diagnostics, there are several paramount challenges that would need to be addressed. In particular, the time required for metabolic labelling of bacterial glycans must be rapid. Further, the delivery and pharmacokinetic properties of the covalent partner need to be optimized. Additionally, any extra steps that have to be performed, such as exposure to light, should be tolerated by patients in a clinical setting. Finally, since some bacteria change their glycan coat to evade host immune detection, the approach could include a panel of sugars to cirvcumvent potential resistance mechanisms. Addressing these constraints would set the stage for looking beyond the scope of bacterial glycans. Indeed, this chemical reporter-based approach has widespread promise for labelling, targeting, and imaging diverse biomolecules in a wide variety of normal and pathological settings.

Acknowledgements

We gratefully acknowledge funding for this work from the Camille & Henry Dreyfus Foundation and by grants from the National Center for Research Resources (5P20RR016463-12) and the National Institute of General Medical Sciences (8 P20 GM103423-12) from the National Institutes of Health. We thank members of the Dube lab for thoughtful comments and discussions.

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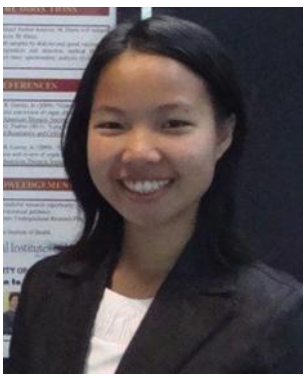

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