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Exterior design: Strategies for redecorating the bacterial surface with small molecules

Samir Gautam^a, Thomas Gniadek^b, Taehan Kim^c, and David A. Spiegel^{c,d,e}

^aDepartment of Cell Biology, Yale School of Medicine, 333 Cedar Street, New Haven, CT 06520

^bDepartment of Pathology, Johns Hopkins School of Medicine, 600 North Wolfe Street, Baltimore, MD 21287

^cDepartment of Chemistry, Yale University, 225 Prospect Street, New Haven, CT 06511

^dDepartment of Pharmacology, Yale School of Medicine, 333 Cedar Street, New Haven, CT 06520

Abstract

Recombinant techniques for expressing heterologous proteins and sugars on the surface of bacteria have been known since the 1980's, and have proven useful in a variety of settings from biocatalysis to vaccinology. The last decade has also seen the emergence of novel methods that allow modification of bacterial surfaces with small, non-biological compounds. Such technologies enable researchers to harness the unique properties of synthetic materials on a live bacterial platform, opening the door to an exciting new set of applications. Here we review strategies for bacterial surface display and describe how they have been applied thus far. We believe that “chemical surface display” holds great potential for advancing research in basic bacteriology and applied fields of biotechnology and biomedicine.

Keywords

Bacterial envelope; Surface display; Surface engineering; Small molecule; Metabolic labeling; Bioorthogonal chemistry

Bacterial surface display

The bacterial envelope is a multilayered organelle that surrounds prokaryotic cells, providing morphological structure, protection from osmotic lysis, and an interface between cell and environment. In Gram-positive organisms, the envelope consists of the plasma membrane and a thick, surface-exposed peptidoglycan cell wall, while Gram-negative organisms possess an additional outer membrane that surrounds the cell wall (Fig. 1).⁽¹⁾ Envelope structures have been intensely studied for over 60 years due to their clinical importance as antibiotic targets, their interactions with the human immune system, and their fascinating cell biology. The biology of peptidoglycan has especially intrigued basic

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^eTo whom correspondence should be addressed. david.spiegel@yale.edu; Phone: (203) 432-8697; Fax: (203) 432-6144 .

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researchers, in part due to its ability to provide incredible rigidity (withstanding osmotic pressures up to 450 lb/in²) and yet maintain sufficient plasticity to allow bacterial growth and division.(2, 3)

With advances in genomics and recombinant DNA techniques over the past few decades, it has become possible to express a diverse array of heterologous proteins and glycans on the bacterial cell surface. Since the first examples of “bacterial surface display” in 1986,(4, 5) this methodology has been applied in a variety of settings including environmental remediation,(6) biofuel production,(7) biocatalysis,(7) biosensing,(8) protein library screening,(9) cancer therapeutics,(10) vaccinology,(11-13) and anti-diarrheal probiotic therapy.(14)

Genetic surface display technologies, however, have one critical drawback: they offer no way of mounting non-genetically encoded molecules on the cell surface. The list of such molecules includes many lipids and carbohydrates, as well as the vast panoply of non-biologic small molecules: fluorophores, affinity tags, spectroscopic probes, crosslinking agents, cytotoxic drugs, vaccine adjuvants, etc. Advances in the field of chemical biology during last decade have begun to address this limitation. A spate of techniques have now been developed for modifying surfaces with non-native synthetic compounds in bacteria, fungi and mammalian cells,(15, 16) as well as in viral,(17, 18) teleost,(19) and even murine (20) systems. This emerging technology of “chemical surface display”, which enables presentation of myriad small molecules on cell surfaces, has already found a number of interesting applications in bacteria, including directed evolution,(21) proteomic analysis,(22) immunotherapy,(23, 24) as well as basic studies on cell wall biology (25) and host-pathogen interactions.(26)

Here we elucidate the basic principles of chemical surface display in bacteria and review examples of this technique reported in the literature to date. We then describe the unique biomedical and biotechnological applications that this strategy has made possible, and suggest potential future directions for the field. We believe that as the techniques of chemical display become increasingly familiar to a broader scientific community, their potential to transform basic research, biotechnology, and medicine will continue to grow.

Basic principles in chemical surface display

There are two basic strategies for installing small molecules of interest onto the surface of bacteria: “direct” and “two-step” incorporation (Fig. 1). In the former, exogenous compounds are inserted *directly* into the bacterial envelope, either by non-covalent means, or by incorporation through the cell’s native biosynthetic machinery (metabolic labeling). (27) In metabolic labeling, small molecule-monomer conjugates are assimilated through endogenous metabolic pathways into polymeric structures present on the bacterial envelope (e.g. oligosaccharide, protein, peptidoglycan, etc.). In the case of re-engineering surface glycans, for instance, chemically modified monosaccharides are taken up by bacteria, metabolically incorporated into growing polysaccharides in the cytoplasm, and mounted on the cell surface (Fig. 1, bottom).(28)

Biosynthetic enzymes, however, are often unable to tolerate bulky chemical cargo such as fluorophores, whose molecular weights often exceed 300 Da. In such circumstances, *two-step* labeling strategies must be employed, in which monomers equipped with small, reactive “chemical handles” are first incorporated into surface structures and then used as an attachment point for synthetic compounds of interest (Fig. 1, top). Importantly, the fact that chemical handles are surface-exposed rather than intracellular allows installation of a much greater diversity of compounds, as they are not structurally limited to those able to cross the lipid membrane.

Critical to the success of the two-step labeling process is the structure and reactivity of the chemical handles, which in general must satisfy three criteria.⁽²⁹⁾ First, the modifying functional group must not prevent the monomer from accessing the enzymatic machinery responsible for its incorporation. Many of the biosynthetic enzymes that mediate oligosaccharide, protein, and peptidoglycan synthesis, for example, are located intracellularly, requiring that the modified monomer cross the cell membrane and enter the cytoplasm. Thus, the chemical appendage must be sufficiently hydrophobic to permit diffusion across the membrane or small enough to permit normal uptake through membrane transporters. Second, modifying groups must not impede monomer recognition by biosynthetic enzymes or transfer to the growing polymer. Third, handles must be bioorthogonal; i.e. they must be physiologically non-perturbing, absent from native biological systems, unreactive with endogenous functional groups, and capable of undergoing chemoselective reaction under physiological conditions with complementary functional groups. Examples of frequently-used bioorthogonal reaction pairs include: (i) azides and terminal alkynes (“click” reaction), (ii) azides and phosphines (Staudinger ligation), (iii) aldehydes/ketones and hydrazines or hydroxylamines, and (iv) tetrazines and strained alkenes such as *trans*-cyclooctenes.^(29, 30)

Strategies for chemical surface display in bacteria

In this section we describe several published strategies that have been developed for installing synthetic molecules in the envelope of live bacteria. These methods are organized according to the subcellular target of modification (Figs. 2 and 4). It should be noted that we focus here on strategies for re-engineering bacterial cell surfaces; approaches for the bioorthogonal modification of *intracellular* biomolecules are therefore mostly excluded from our discussion. As investigations of this kind have provided valuable insights into several aspects of bacterial pathogenesis and physiology—including host-pathogen interaction, transcriptional regulation, translation, quorum sensing, and stress responses—we regret not being able to include them herein. Lead references are provided for interested readers.⁽³¹⁻³⁷⁾

Chemical Modification of Surface Glycans

Surface-associated polymeric sugars represent the paradigmatic target of two-step metabolic labeling, being the first (and perhaps most frequent) structure modified through bioorthogonal surface engineering.⁽³⁸⁾ Here, following incorporation of chemical handle-bearing monosaccharides, small molecules of interest are selectively ligated to the modified polymer at the cell surface (Fig. 1, top). Bacterial surfaces contain a great diversity of glycans, including capsular polysaccharides, teichoic acids (in Gram-positive organisms), glycolipids, and glycoproteins. Lipopolysaccharide (LPS), which constitutes much of the extracellular leaflet of the outer membrane in Gram-negative bacteria (Fig. 2a), has been a particularly frequent target in metabolic labeling protocols. LPS consists of three basic components: (i) lipid A, a highly conserved glycolipid that serves as the hydrophobic anchor in the outer membrane (Fig. 3a), (ii) core polysaccharide, which contains two well-conserved 3-deoxy-d-mannooctulosonic acid (KDO) residues and a more variable oligosaccharide region (Fig. 3b), and (iii) O-antigen, which is structurally diverse and can grow to extremely large sizes (N values up to 164 in certain strains of *E. coli*) (Fig. 3c).⁽³⁹⁾

Researchers have disclosed several strategies for re-engineering LPS in live bacteria (Fig. 2a). In a pioneering example of two-step chemical modification, the endogenous synthetic pathway for L-fucose (a component of O-antigen) was replaced with a heterologous L-fucose salvage pathway in *E. coli*, rendering the recombinant strain dependent on exogenous L-fucose for LPS synthesis. Due to the promiscuity of the enzymes in this salvage pathway it was possible to introduce azide-functionalized L-fucose into LPS (Fig. 3a) in the mutant

strain (step 1 – metabolic labeling). The resulting surface-exposed azide moieties were then detected through click reaction with an alkynyl-fluorescein derivative (step 2 – bioorthogonal ligation with a chemical probe).⁽⁴⁰⁾ Introduction of unnatural sialic acids into the LPS core oligosaccharide of *H. ducreyi* has also been demonstrated,⁽⁴¹⁾ although the sugar analogs used in the study lacked the reactive “handles” necessary for further modification. Very recently, a method for labeling the LPS core in wild type bacteria was established using an azide derivative of KDO, which was assimilated into *E. coli* LPS and detected via click reaction with an alkyne-derivatized fluorophore (Fig. 3b).⁽²⁸⁾ The group further showed KDO incorporation in several Gram-negative species, suggesting the method is both broadly applicable and straightforward, as it does not require genetically manipulated bacterial strains.

Chemical glycoengineering in bacteria has extended beyond LPS to glycoproteins as well (Fig. 2b). Using alkyne-bearing fucose derivatives, the glycoproteomes in several *Bacteroidales* species were labeled and then ligated through click reaction with azide-containing chemical probes.⁽⁴²⁾ Similarly, azidosugars were used to label glycoproteins in *H. pylori*, although detection of azide-modified proteins in this study was performed not in live cells, but cell lysates.⁽⁴³⁾ Finally, it was shown that the heavily glycosylated flagellae of *C. jejuni* could be re-engineered with azide moieties using a membrane-permeable azide-bearing precursor to pseudaminic acid—a nine-carbon sugar that is appended to flagellin proteins in several Gram-negative pathogens.⁽⁴⁴⁾ An alkynyl-biotin probe was clicked onto modified pseudaminic acid *in vivo*, and used to isolate the tagged glycoprotein to confirm its identity as a flagellin.

Chemical Modification of Surface Proteins

Surface proteins have also been used as scaffolds for the attachment of small molecules to bacteria through two-step metabolic labeling (Fig. 2c).⁽³¹⁾ In analogy to the modified monosaccharides used to metabolically label glycans, noncanonical amino acids (ncAAs) are incorporated into proteins and presented on the cell surface for subsequent ligation. In *E. coli*, for instance, it was shown that the ncAA azidohomoalanine serves as a methionine surrogate, replacing the natural amino acid during translation through recognition by the methionine tRNA-synthetase, ligation to tRNA^{Met}, and ribosomal incorporation into proteins. Presence of azidohomoalanine in extracellular loops of the outer membrane protein OmpC was demonstrated through click reaction with an alkynyl-biotin derivative, which was subsequently used to recruit fluorescent streptavidin to the cell surface.⁽⁴⁵⁾ In later studies it was discovered that the native translational machinery in *E. coli* is also able to replace methionine with azidoalanine, as well as with larger amino acids such as azidonorvaline and azidonorleucine.⁽⁴⁶⁾ Labeling efficiency was shown to be maximized in methionine auxotrophs; i.e. strains lacking the metabolic capacity to synthesize methionine *de novo*. ncAA surface display has also been used in directed evolution as a means to select for tRNA-synthetase mutants optimized for recognizing handle-bearing ncAAs and ligating them to cognate tRNAs.^(21, 47)

It should be emphasized that in this approach, genetically encoded methionines are globally replaced with ncAAs. This metabolic manipulation affords advantages in some settings (e.g. proteomic analysis), but it also precludes interrogation of individual gene products. For such investigations, an alternative method has been developed in which a novel tRNA-tRNA synthetase pair—designed to incorporate a ketone containing ncAA (*m*-acetyl-l-phenylalanine) into recombinant proteins in response to the amber nonsense stop codon—is introduced into *E. coli*. Using this genetically-modified strain, it has been shown that the ketone functionality can be site-specifically introduced into surface proteins (by inserting the amber codon into desired positions of the recombinant gene sequence), and detected *in vivo* using hydrazide-functionalized fluorophores.⁽⁴⁸⁾

Chemical Modification of Surface Lipids

Two recent studies describe metabolic labeling of lipids in mycobacteria, a genus that includes *M. tuberculosis* (*MTB*) and *M. leprae*, the etiological agents in tuberculosis and leprosy, respectively. *MTB* claims approximately two million lives per year and is estimated to latently infect one-third of the world's population.(49) Mycobacteria, which are classified as Gram-positive based on DNA-based molecular taxonomy, possess a characteristically thick cell wall and lack an outer membrane, but their cell envelope is different from ordinary Gram-positive envelopes in a number of ways (Fig. 5).(49) Extending outwards from the peptidoglycan wall is a unique polysaccharide layer consisting of arabinogalactan, which in turn serves as an attachment point for a diverse set of mycobacterial fatty acids known as mycolic acids. Intercalated into the alkyl chains of mycolic acids are a number of surface lipids, which include trehalose dimycolate (TDM, also called cord factor), a glycolipid consisting of the disaccharide trehalose esterified with two complex fatty acids (Fig. 5b). TDM confers several clinically important properties upon the mycobacterial envelope, including increased pathogenicity and impermeability to many hydrophilic antibiotics; it is therefore an important subject of study.(49)

Display of small molecules on TDM has been achieved both through direct and two-step labeling strategies. In an example of the former, fluorescein-derivatized trehalose analogs were found to be taken up by *MTB*, esterified with the complex fatty acid mycolate, and inserted as fluorescent TDM derivatives into the outer membrane (Fig 5a). Labeling with these substrates was made possible by the remarkable tolerance of TDM biosynthetic enzymes to bulky substituents. This methodology has enabled researchers to fluorescently label *M. tuberculosis* not only in culture, but also within the phagosome of infected macrophages, thus providing a novel system for studying the interaction between the human host and this important pathogen.(26)

In a two-step method of modification, TDM and related trehalose-containing metabolites (the mycobacterial “trehalome”) were metabolically labeled with azide-functionalized trehalose analogues and ligated using click chemistry in *MTB*, *M. smegmatis*, and the attenuated strain *M. bovis*, which is used in the Bacillus Calmette–Guérin TB vaccine (Fig. 5c).(50) The precise metabolic pathways of incorporation in these experiments (which included both the antigen 85 and recycling pathways) were determined using deletion mutants, and were shown to be tolerant to trehalose derivatives bearing azide moieties at any position on the sugar. Biochemical analysis and molecular imaging studies confirmed specific labeling of TDM and related metabolites.

Chemical Modification of Peptidoglycan

The majority of examples of chemical display in Gram-positive bacteria involve incorporation of non-native molecules into the surface-exposed peptidoglycan cell wall (Fig. 4). In an early example of two-step labeling, ketone-bearing derivatives of the murein monomer, UDP MurNac pentapeptide (l-alanyl- γ -d-glutamyl-l-lysyl-d-alanyl-d-alanine, a building block of peptidoglycan), were installed in the cell wall of lactic acid bacteria and detected using hydrazide-functionalized fluorophores (Fig. 4a).(51, 52) Using a fluorescein-conjugated derivative of this murein monomer, the same research group was able to demonstrate direct incorporation of chemical cargo into the cell walls of both Gram-positive and Gram-negative bacteria (Fig. 2d), although in the latter case, calcium chelation was necessary to increase permeability of the outer membrane to exogenous compounds. In a follow-up study, ketone groups installed through the same method were shown to react with aminoxy-functionalized oligomannose, promoting adhesion to a lectin-coated surface.(53) Recently, an improved labeling method was reported, which utilizes derivatives of N-acetylglucosamine—a peptidoglycan precursor molecule that is much simpler than UDP-

MurNAc pentapeptides (Fig. 4b). This streamlined system permitted robust surface display of ketones, reaction with biotin-hydrazide conjugates, and recruitment of fluorescent streptavidin in a number of Gram-positive species.⁽⁵⁴⁾ Finally, in a related approach, it was found that Gram-negative bacteria were able to take up and incorporate a fluorescein-modified version of the tripeptide L-alanyl- γ -D-glutamyl-L-lysine, a natural product of peptidoglycan turnover.⁽⁵⁵⁾ This direct labeling technique allowed microscopic investigation of peptidoglycan synthesis and turnover in relation to cell growth and division in *E. coli*.

A recent report has described a new method of peptidoglycan modification that exploits an endogenous mechanism present in both Gram-positive (Fig. 4c) and Gram-negative species (Fig. 2e) for incorporating noncanonical D-amino acids (such as D-alanine, -glutamic acid, -aspartic acid, and -serine) into the cell wall.⁽⁵⁶⁾ This study showed that the cell wall biosynthetic machinery underlying this mechanism can tolerate synthetic D-amino acids functionalized with small chemical handles such as alkynes and azides, as well as with bulkier groups like fluorophores.⁽⁵⁷⁾ This labeling technique enabled live three-color super-resolution imaging of cell wall structure and dynamics in a phylogenetically and morphologically diverse set of bacteria. Incorporation of fluorophores was found to occur specifically at sites of new cell wall synthesis, such that the redistribution of pulsed fractions of peptidoglycan could be tracked in real time.⁽⁵⁷⁾

Direct modification of the Gram-positive cell wall has also been accomplished by hijacking the function of sortase A, a biosynthetic enzyme on the extracellular face of the plasma membrane that mediates covalent attachment of proteins to peptidoglycan. Sortase A recognizes the pentapeptide motif LPxTG in secreted proteins (where x can be any amino acid), cleaves between the threonine and glycine, and transfers the LPxT peptide along with its N-terminal protein cargo to the peptidoglycan cell wall.⁽⁵⁸⁾ Thus, incubation of bacteria with LPxTG peptides carrying N-terminal chemical cargo leads to efficient chemical modification of the cell wall (Fig. 4d).⁽⁵⁹⁾ This method benefits from the fact that the motif recognized by sortase A is remote from the conjugated synthetic molecule, permitting direct incorporation of relatively large compounds such as fluorophores.

Chemical Modification of Lipid Membranes

While the preceding techniques rely on covalent modification of surface structures with exogenous molecules, another set of methods has been devised for non-covalently attaching small molecules to the cell envelope through association with the membrane. In one such example, carrier peptides have been shown to bind to the surface membrane of bacterial magnetite particles (BMPs)—derivatives of magnetic bacteria consisting of magnetite (Fe₃O₄) granules enveloped in an inner membrane-derived lipid bilayer (Fig. 2f).⁽⁶⁰⁾ Cationic peptides conjugated to organic dyes were found to insert into the BMP membrane. While this study utilized BMPs as opposed to intact bacteria (a necessity, in fact, because the peptide possessed antimicrobial properties), it nevertheless suggests the feasibility of using membrane-resident peptides to incorporate bulky chemical conjugates into bacterial surfaces—an attractive alternative to metabolic labeling techniques, which may be less tolerant to large substituents.

Similarly, substituted lipids have been used to label membranes non-covalently, thus enabling interrogation of membrane lipid distribution and diffusion. Lipids conjugated both to fluorophores and photoactivatable cross-linkers have been utilized as probes in both Gram-positive (Fig. 4e) and Gram-negative bacteria (Fig. 2g), as discussed extensively elsewhere.⁽²⁵⁾ These conjugates represent yet another valuable means of installing chemical moieties onto the cell surfaces, and carry the particular advantage—like cationic peptides—of non-specific association with membranes, which allows generalization of the technique

across bacterial genera and easy installation of bulky chemical cargo. Of course, in applications where selectivity is critical (e.g. detection of Gram-positive bacteria in diagnostic samples),(61) as described in the following section), such non-specific labeling techniques are less desirable.

Chemical Modification of Surface Structures Using Antibiotics

Although antibiotic conjugates are often highly toxic to bacteria, they have nevertheless proven useful in select bacteriological and biotechnological applications (Fig. 4f). Fluorescent antibiotics, for instance, have provided crucial insights into the basic biology of cell walls in microscopic investigations, as reviewed elsewhere.(25) Antibiotic conjugates have also been utilized in immunotherapeutic applications. In one example, it was shown that multivalent vancomycin–fluorescein conjugates could be installed on the surface of several Gram-positive pathogens through binding to *D*-alanyl-*D*-alanine moieties within peptidoglycan. Fluorescein, used in this study as a hapten, effectively recruited anti-fluorescein antibodies to the bacterial surface, resulting in opsonophagocytosis by macrophage cell lines.(23) In an extension of this approach, a bacteria-targeting ligand (vancomycin) and a phagocyte-targeting ligand (Fc portion of IgG) were conjugated to a polylysine/polyethylene glycol-based polymer to create an artificial multivalent opsonin that similarly augmented phagocytosis of Gram-positive bacteria.(24) Antibiotic conjugates have also been used in novel diagnostic methods. For example, Gram-positive bacteria were selectively labeled (non-covalently) using *trans*-cyclooctene-bearing derivatives of vancomycin and daptomycin, and detected via bioorthogonal reaction with tetrazine-functionalized magnetofluorescent nanoparticles.(61)

These findings highlight some the unique properties of antibiotics that may be leveraged in surface display, namely their exquisite specificity for cell surface targets such as cell wall structures and biosynthetic enzymes, and their broad applicability across bacterial genera. In addition to vancomycin, the antibiotics ramoplanin, which binds lipid II (an intermediate in peptidoglycan synthesis),(62) and bacitracin, which covalently attaches to penicillin binding proteins,(63) are frequently used as “Trojan horses” for the delivery of small molecules to specific sites in the cell wall.(25)

Applications of chemical surface display in bacteria

Several applications of chemical surface display in bacteria have been demonstrated thus far, including directed evolution,(21) proteomic analysis,(22) immunotherapy,(23, 24) molecular imaging,(25) and studies on host-pathogen interactions.(26) Here we describe how chemical display has been employed so far in biomedical and biotechnological settings, and propose additional ways it might find use in the future (Fig. 6). In assembling this list of potential applications, we have taken cues from the comparatively mature study of chemical modification in non-bacterial systems (such as mammalian cells and fungi), which has reached a far greater breadth of implementation.

Before proceeding, it must be recognized that since exogenously-applied chemicals are not genetically encoded, they do not regenerate; therefore incorporated compounds are gradually lost through dilution with passing cell divisions. Due to this transience of labeling, chemically-modified organisms will likely be ineffective in applications requiring propagation outside the laboratory (such as bioremediation, for example), for which genetically re-engineered bacteria, in contrast, are well-suited.(6) On the other hand, chemical display methods are advantageous in that they circumvent risks associated with genetically modified organisms (GMOs), such as the spread of antibiotic resistance genes, reversion to virulence of attenuated vaccine strains, and release of organisms with a fitness

advantage, which may lead to unrestrained propagation and damaging environmental consequences.(64)

Imaging

Perhaps the best-explored application of chemical display to date is molecular imaging with organic fluorophores (Fig. 6a).(25, 65) Compared to the current “gold standard” of labeling biomolecules for live cell microscopy—genetic fusion with fluorescent proteins (FPs)—bioorthogonal tagging with chemical dyes offers several advantages.(66) First, the utility of FPs as direct reporters of biomolecule localization is limited to proteins (as they alone are genetically encoded), rendering the remaining classes (oligosaccharides, lipids, peptidoglycan, etc.) largely intractable to real time imaging. Site-specific chemical incorporation of fluorophores via metabolic labeling, however, provides a powerful means of observing these non-genetically encoded molecules in live cells. Second, in comparison to FPs, which are relatively large (~30 kDa) and prone to aggregation, organic dyes are minute (~0.5 kDa) and comparatively resistant to multimerization.(65) These physical properties allow accurate measurements in biophysical experiments and help to reduce artifacts in imaging applications. The danger of such artifacts is highlighted by a recent study which revealed that the helical localization of the bacterial cytoskeletal protein, mreB, described in a series of influential reports (67, 68) was attributable to an FP tag rather than the physiology of the native protein.(69) Third, small molecule fluorophores have significantly narrower excitation/emission spectra than FPs, facilitating simultaneous imaging of >3 colors. Finally, the photophysical performance of organic dyes far outstrips that of FPs both in terms of brightness and photostability. These properties enable the use of recently-developed super-resolution microscopy techniques such as stochastic optical reconstruction microscopy (STORM) and 3D structured illumination microscopy (3D-SIM), which depend on high quality fluorophores.(70) In the study of bacteria, which have dimensions in the range of a single micron (the average diameter of *S. aureus*, for instance, is 800 nm), the ability of these microscopy techniques to break the ~300 nm limit of resolution in the lateral dimension is of particular value.(70)

Here it is worth noting a study on a bacterial metabolite that contains a chemical moiety rarely seen in biological systems (the isonitrile group) for its potential to be used as an optically active reporter in chemical surface display. It was recently shown that rhabduscin—a metabolite produced by insect pathogens that functions as an inhibitor of phenoloxidase (a key component of the insect’s innate immune system)—can be detected at the surface of rhabduscin-producing bacteria.(71) One of the methods used for detection involved Raman spectroscopy, which is able to localize isonitrile moieties due to the characteristic Raman resonance signal of this group. This study underscores the potential utility of isonitrile functionalization in metabolic labeling; it is extremely small (26 Da), can react bioorthogonally with tetrazines,(72) and is compatible with optical methods such as imaging and flow cytometry due to its Raman activity.(73) This latter property may be particularly useful in settings where the size of organic fluorophores (often greater than ten times that of the isonitrile function) is problematic. Alkynes can also be useful along these lines given that the alkyne’s Raman absorption (2120 cm^{-1}) lies within the Raman-silent region of cells ($1800\text{--}2800\text{ cm}^{-1}$) and is almost identical to that of isonitrile ($2,121\text{ cm}^{-1}$). Indeed, DNA labeled with alkyne-containing thymidine analogs has been visualized in live mammalian cells using Raman microscopy.(74)

Proteomic and glycomic analysis

Chemical display has been used extensively in non-bacterial systems for the purpose of “omic” analysis of surface biomolecules. A common scheme involves incorporation of affinity tags such as biotin into surface structures, lysis of labeled cells, affinity purification

(e.g. with streptavidin), and gel- and/or mass-spectrometric-based analytical profiling (Fig. 6b). For example, this technique has recently been used for comprehensive mapping and identification of glycosylation sites in human prostate cancer cells.(75)

An analogous strategy has recently been translated into bacteria for the characterization of the *E. coli* lipoproteome. After incorporation of alkyne-functionalized fatty acids into lipoproteins, cell lysates were allowed to react with an azido-biotin conjugate, lipoproteins were purified using streptavidin beads, and the isolated proteins were analyzed via mass spectrometry.(22) In addition, using non-specific labeling of surface proteins with NHS-biotin, followed by streptavidin-based affinity purification, previously unknown surface proteins (including potential vaccine antigens) were identified in *Leptospira* species.(76) The high efficiency of this approach allowed proteomic profiling of relatively small bacterial samples, thus enabling characterization of bacterial surface expression during infection through analysis of clinical isolates.(76) Studies like these may help to identify bacterial surface proteins that contribute to bacterial virulence and/or serve as target antigens for vaccine development.(77) Such chemistry-based proteomic approaches have the potential to complement genetic methods such as reverse vaccinology, which utilizes genomic data and predictive algorithms for the *in silico* identification of surface proteins.(78) Yet another application of bacterial chemical display might involve metabolic incorporation of peptidoglycan precursors equipped with photo-crosslinking handles (e.g., diazirines or benzophenone analogs) into the cell wall. Following installation, such chemical crosslinkers could be activated to react with adjacent proteins, facilitating discovery of antibiotic targets within the cell wall.(55)

Interrogating individual proteins of interest

In contrast to “shotgun screening” approaches, in which entire classes of macromolecules are tagged for analysis, site-specific chemical modifications have been used to investigate the function of individual proteins in complex cellular systems.(16) Such strategies have included: (i) the insertion of ncAA’s at defined genetic positions via heterologous translation machinery;(16) (ii) the use of formylglycine-generating enzyme which convert specific cysteine residues into aldehyde-functionalized formylglycine motifs for subsequent ligation;(79) (iii) the conjugation of alkyl azides to a specific 22 amino acid acceptor peptide by the enzyme lipoic acid ligase;(80) (iv) the attachment of the biotin motif to a 15 amino acid consensus sequence using biotin ligase enzymes;(81) (v) the orthogonal tagging of proteins of interest with the enzyme hAGT, which can be labeled with guanine-probe conjugates (a strategy termed “SNAP-tagging”);(82) and (vi) the fusion of proteins of interest to dihydrofolate reductase (DHFR), which binds with high selectivity to synthetic trimethoprim-probe conjugates.(83) These techniques can be exploited for a wide range of applications including microscopic localization of bacterial proteins examination of receptor-ligand interactions, single protein purification, and many others.(84)

Immunological applications

Some of the most clinically impactful applications of chemical surface display fall within the sphere of immunology. As shown above in the example of selective fluorescent labeling of surface lipids in *M. tuberculosis* within macrophage phagosomes,(26) insight may be gained into host-pathogen interactions through the use bioorthogonal chemical probes. One can envision this paradigm extending beyond the cellular level as well, perhaps through attachment of spectroscopic probes to the surface of pathogens to track their fate *in vivo* in animal models of infection. The use of vancomycin conjugates to promote phagocytosis of Gram-positive pathogens(23, 24) also points the way to novel immunotherapeutics that may one day help to contain bacterial disease (Fig. 6c). Finally, an unexplored direction that

merits study is the modification of bacterial surfaces with chemical adjuvants to create novel whole cell vaccines (Fig. 6e).

Clinical diagnostics

The high specificity of certain labeling methods for unique species of bacteria might also enable their use as diagnostic tools (Fig. 6d). For instance, as described above, *trans*-cyclooctene-containing derivatives of vancomycin bind specifically to *d*-Ala-*d*-Ala-motifs in Gram-positive bacteria, allowing their rapid detection in clinical isolates via ligation with tetrazine-functionalized magnetofluorescent nanoparticles.(61) Alternatively, by using a *trans*-cyclooctene-modified version of crystal violet – a dye used in the common Gram stain – can serve as a bio-orthogonal handle for attaching tetrazine-containing magnetofluorescent particles.(85) Such particles enable identification of labeled bacteria via fluorescence microscopy or micromagnetic resonance (μ NMR). The enhanced sensitivity of these methods compared with light microscopy obviates the need for *in vitro* culture and sample purification, potentially enabling direct, automated diagnosis in point-of-care settings. Labeling-based microbe identification may also prove orthogonal and complimentary to emerging nucleic acid-based molecular speciation tests.(86).

Miscellaneous

One potential application of chemical surface display that has not yet been explored is the installation of fluorescent sensors capable of detecting cellular analytes such as ions, metabolic intermediates, or signaling molecules. Such an approach may afford insights into the biochemical milieu of the bacterial envelope at unprecedented resolution.(66, 87) Another possibility involves incorporating bio-orthogonal reactive groups at chemically defined sites within the envelope, which could enable recapitulation of three-dimensional cellular structures through chemically-mediated cell-cell interactions.(88) It might even be conceivable to use such chemistry to control the assembly of synthetic biofilms.(89) Such studies may reveal valuable details about the biology of these structures, which are of clinical importance due to their role in the pathogenesis of bacterial infection.(90)

Conclusion and outlook

An impressive suite of chemical display techniques has been developed, enabling researchers to capitalize on the unique structures and reactivities of chemical compounds such as fluorophores for imaging studies and affinity reagents for proteomic analysis. We anticipate that the number of chemical display techniques is likely to increase in the coming years as the generalizable principles of metabolic labeling are applied to different species and surface biomolecules. Furthermore, combining chemical techniques with well-established genetic methods has the potential to deliver even more sophisticated paradigms that are able to simultaneously leverage the biological robustness of the bacterial vector and the chemical versatility of synthetic compounds, with far-reaching impact in basic bacteriology, biotechnology, and medicine.

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Highlights

- New methods allow installation of small molecules on the surface of live bacteria
- Bacterial chemical display enables applications that were not previously possible
- Fields of basic research, biotechnology, and medicine have already felt its impact

Figure 1.

Two-step and direct metabolic labeling. This schematic depicts bacterial uptake of chemically derivatized monosaccharides (indicated in blue), metabolic incorporation into a growing oligosaccharide (indicated in gray), and export of the modified oligosaccharide to the cell surface. In two-step labeling (top), the monosaccharide is modified with a chemical handle (represented here as an azide), to which a fluorophore-alkyne derivative may be bioorthogonally ligated after surface exposure. In direct labeling (bottom), the chemical cargo (represented here as a green fluorophore) is attached to the exogenous monosaccharide and is thus installed directly onto the cell surface.

Figure 2.

Chemical surface display methods in Gram-negative bacteria. Exogenously delivered molecules (indicated in blue) are conjugated to a small molecule (SM) of interest, which we depict here as a fluorophore (indicated in green). **(a)**, SM-monosaccharide incorporated into lipopolysaccharide. **(b)**, SM-monosaccharide within surface proteoglycans. **(c)**, Incorporation of SM-noncanonical amino acid conjugates into an extracellular loop of a surface protein. **(d)**, Outer membrane-associated SM-lipid conjugates. **(e)**, SM-UDP MurNac peptide conjugates assimilated into peptidoglycan. **(f)**, SM-D-amino acid conjugates incorporated into peptidoglycan. **(g)**, Cationic peptides conjugated to SM inserted into the outer membrane. Abbreviations: IM, inner membrane; OM, outer membrane; PG, peptidoglycan; PM, plasma membrane.

Figure 3.

Metabolic labeling of lipopolysaccharide. The predominant lipopolysaccharide (LPS) glycoform expressed in *E. coli* O157:H7 is represented here. (91) (a), Lipid A anchors LPS to the outer membrane. (b), A more conserved portion of the core oligosaccharide has been labeled with azide-containing KDO (blue). (c), The highly variable O-antigen has been labeled with azide-containing L-fucose (indicated in blue). Abbreviations: EtN, ethanolamine; GalNAc, D-N-acetylgalactosamine; Glc, D-glucose; GlcNAc, D-N-acetylglucosamine; Hep, L-glycero-D-manno-heptose; KDO, 3-deoxy-D-manno-octulosonic acid (2-keto-3-deoxyoctulosonic acid); L-Fuc, L-fucose; PerNAc, D-N-acetylperosamine; PO₄, phosphate.

Figure 4. Chemical surface display methods in Gram-negative bacteria. Exogenously delivered molecules (indicated in blue) are conjugated to a small molecule (SM) of interest, which we depict here as a fluorophore (indicated in green). **(a)**, SM-UDP MurNac peptide conjugates incorporated into peptidoglycan. **(b)**, SM-GlcNac conjugates assimilated into peptidoglycan. **(c)**, SM-D-amino acid conjugates incorporated into peptidoglycan. **(d)**, Sortase A-mediated attachment of SM-LPxTG peptide conjugates into peptidoglycan. **(e)**, Plasma membrane-associated SM-lipid conjugates. **(f)**, SM-Vancomycin conjugates bound to d-alanyl-d-alanine in the cell wall as an example of antibiotic-based labeling methods. Abbreviations: PG, peptidoglycan; PM, plasma membrane.

Figure 5.

Structures of the mycobacterial cell envelope and trehalose dimycolate (cord factor). Mycobacteria possess a thick cell wall, arabinogalactan polysaccharides, and a layer of mycolic acids into which surface lipids intercalate. One particularly abundant surface lipid is trehalose dimycolate (TDB), which has been metabolically labeled through both direct and two-step methods (a). Highlighted in the inset is the structure of native TDB (b) and a modified version of the glycolipid (c) containing an azido-trehalose moiety (indicated in blue), which permits two-step labeling.

Figure 6.

Applications of chemical surface display in bacteria. **(a)**, Through site-specific attachment of organic fluorophores, the localization of surface structures and enzymatic activity may be visualized using microscopy. Here a couplet of *Staphylococcus aureus*, labeled in three colors and imaged with 3-dimensional structured illumination, is shown (our unpublished data). **(b)**, Incorporation of affinity probes such as biotin allows proteomic analysis of surface biomolecules through techniques such as two-dimensional electrophoresis (left) and mass spectrometry (right). **(c)**, Groups have shown that surface-bound haptens and opsonins can promote opsonophagocytosis of bacteria. Such approaches may lead to the development of novel anti-bacterial immunotherapeutics. **(d)**, Species-specific labeling with magnetofluorescent particles offers a novel means of diagnosing bacterial infections in clinical samples. **(e)**, Incorporation of chemically defined adjuvants into the envelope of pathogenic bacteria may lead the way to new whole cell vaccines.