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Small-molecule fluorescent probes: big future for specific bacterial labeling and infection detection

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Bacterial infections remain a global healthcare problem that is particularly attributed to the spread of antibiotic resistance and the evolving pathogenicity. Accurate and swift approaches for infection diagnosis are urgently needed to facilitate antibiotic stewardship and effective medical treatment. Direct optical imaging for specific bacterial labeling and infection detection offers an attractive prospect of precisely monitoring the infectious disease status and therapeutic response in real time. This feature article focuses on the recent advances of small-molecule probes developed for fluorescent imaging of bacteria and infection, which covers the probe design, responsive mechanisms and representative applications. In addition, the perspective and challenges to advance small-molecule fluorescent probes in the field of rapid drug-resistant bacterial detection and clinical diagnosis of bacterial infections are discussed. We envision that the continuous advancement and clinical translations of such a technique will have a strong impact on future anti-infective medicine.

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1. Introduction

Bacteria, both pathogenic and nonpathogenic, are ubiquitous in the environment, exposing human beings to countless different species daily.[1] Of the non-pathogenic bacteria, the mammalian gut microbiota particularly play crucial roles in gut development, nutrient production, regulation of biological metabolism and immune system.[2–6] On the other hand, pathogenic bacteria strains are harmful to public health, and can lead to bacteria-derived diseases, such as foodborne illnesses, respiratory tract infection, urinary tract infections, and healthcare-associated infections.[1,7–9] Despite the multitude of available antibiotics, bacterial infections remain a major healthcare threat due to the spread of antibiotics resistance and evolving pathogenicity.[10–13] Fortunately, most infectious diseases can be treated and cured if diagnosed accurately at an

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early stage, when the infection has not developed into a systemic level and causes irreversible damage.[14,15]

Traditional diagnostic approaches used for bacterial infection in clinics mainly rely on blood assays, *e.g.*, C-reactive protein (CRP), procalcitonin test (PCT), and/or *via* anatomically nuclear imaging, *e.g.*, X-ray, computed tomography (CT), positron emission tomography (PET) and magnetic resonance imaging (MRI).[16,17] Although these techniques hold great merits, some limitations such as radiation concerns, complicated operation, expensive equipment and unsatisfactory precision still exist in practical use.[18,19] Optical (fluorescent) imaging is an alternative solution for the early detection of bacterial infections, showing improved sensitivity and accuracy for targeted bacterial labeling, as well as infection sensing.[14,19–21] Within such a unique imaging modality, bacterial pathogens or infection-associated biomarkers/pathways are precisely visualized using fluorescent agents that can specifically respond with the target of interests, thus offering a safe, noninvasive, time/cost-effective and more flexible molecular imaging with high resolution to benefit infection disease theranostics.[14] Despite the great promise, fluorescent imaging is currently mainly used in oncology (*e.g.*, image-guided tumor sensing and surgery) instead of human infection diagnostics, which has not yet been widely applied within the clinical setting, on account of either the inherent issues of light-tissue interactions (*e.g.*, reflection, scattering and absorption) or a lack of clinically feasible imaging agents and instrumentations.[22]

Owing to recent advances in both fluorescent molecule development and imaging setups upgrade, most of the limitations encountered in optical imaging can now be minimized and even addressed, and continuous efforts towards bacterial infection detection *in vivo* are producing a variety of novel fluorescent probes. For example, the fluorophores working in the near infrared (NIR, 700–900 nm) optical window have been validated to be promising for clinical translations. This is because the NIR imaging with reduced tissue absorbance and scattering allows for more sensitive signal readout in deep tissues.[23–27] Moreover, the US Food and Drug Administration (FDA) clinically approved NIR dye, indocyanine green (ICG), has been extensively used in humans, *e.g.*, for tumor surgery.[28,29] In recent decades, several types of fluorescent molecular probes have been conducive to the progression of specific bacterial infection detections. Moreover, some initial probe design strategies, including bacteria-targeted labeling, bacteria-activatable and/or infection-responsive sensing systems on the basis of fluorescence resonance energy transfer (FRET), photo-induced electron transfer (PET), and charge transfer luminescence mechanisms, have also been developed.[14,21,30] So far, the fluorescence imaging of bacteria and infections is steadily moving forward in clinics. The further progress of the medical implementations will ultimately be promising for the inspection of bacterial infections in the human body.

In this feature article, we will mainly focus on the advances of small-molecule fluorescent probes developed for bacteria and infection imaging. The fluorescent probe design principles, especially for bacterial recognitions are first discussed in detail, followed by highlighting the representative results and applications in bacterial imaging and infection sensing. Additionally, the perspectives and challenges to advance small-molecule fluorescent probes in the field of rapid drug-resistant bacterial detection and clinical diagnosis of bacterial infections are presented in the end.

2. The design of small-molecule fluorescent probes

2.1 Small-molecule fluorophores

Considerable efforts have been devoted to the design of miscellaneous fluorogenic structures with the aim to fabricate novel small-molecule fluorescent probes for advanced industrial and bio-analysis applications.[31,32] The highly tunable emissions of small organic molecules are only determined by their chemical structures and less dependent on the size, which is different from inorganic or polymer-based nanoplatforms.[33] In addition, small-molecule dyes are particularly efficient and safe in the chemical and biological detection, owing to the high quantum yields and good biocompatibility.[34] So far, a wide variety of fluorescent dyes, such as rhodamines, bodipy, cyanines, squaraines and others are available commercially and/or in research. These fluorophores range from the visible (400–700 nm) to near infrared (NIR, 700–900 nm) regions of the electromagnetic spectrum. While shifting the optical spectra of fluorescent dyes towards the NIR window is promising for enabling sensitive bioimaging in deep tissues, other factors for fluorophores advancement need to be considered to make them more usable, such as the greatly improved water solubility, photo-stability, quantum efficiency, ease of modification, biocompatibility, and so forth.[20,25,35,36]

2.2 Design principles of fluorescent probes

The application of fluorophores for biomedical sensing depends on a sensitive signal change (on/off) in the fluorescence, which requires unique interactions between the analytes and fluorescent molecules.[37] Moreover, the highly complex biological environment can affect the photo-physical process of the fluorophores and lead to background interference.[38] Alternatively, a more versatile and reliable signal readout can be achieved by the use of fluorescent probes. Such strategy needs rational modification of the common fluorophores with complementary components addition.

As illustrated in Fig. 1, the design of small molecular fluorescent probes can be mainly divided into two categories, termed as targeted and responsive (or activatable) systems. In general, targeted probes rely on the specific fluorescence contrast distribution or accumulation upon interaction with the target of interests. Given the ''always on'' fluorescence of fluorophores upon light excitation, the effective strategy for improving the signal-to-noise ratio (SNR) of these molecules is by conjugation with targeting ligands, *e.g.*, small molecules, oligosaccharides, peptides, proteins, antibodies, and aptamers.[39,40] Despite the great promise of ligand-mediated imaging and therapy, the current ''always-on'' molecular probes remain restricted by the nonspecific signal readout and deficiency of quantitative sensing

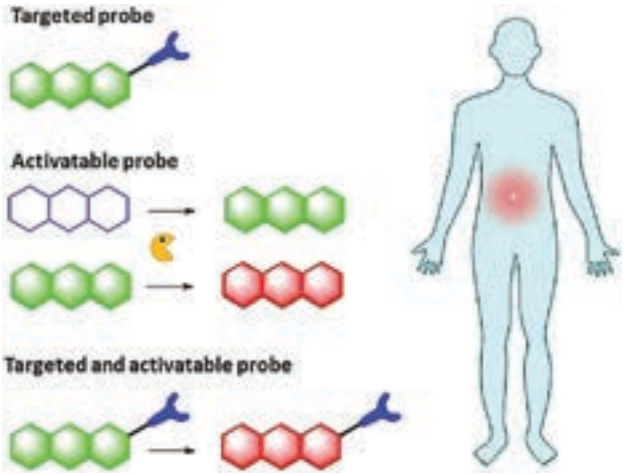

Fig. 1 Illustration of the design strategies of small-molecule fluorescent probes for specific targeting and labeling.

capability.[41] Another type of design is the responsive system that the intrinsic fluorescence signal of fluorophores can only be activated by the sensing target or disease micro-environment, thereby allowing for higher SNR and lower limit of detection (LOD) in comparison with the "always on" targeted probes.[42,43] Furthermore, such activatable probes provide the capability for the quantitative sensing and imaging of biological analytes. Typically, the design of activatable molecular probes undergoes two strategies based on either the stimuli-responsive emission enhancement (turn-on type) or emission color changes (ratiometric type).

The main principle of such activatable probes design usually involves Förster (or fluorescence) Resonance Energy Transfer (FRET), photo-induced electron transfer (PET), and charge transfer luminescence mechanisms.[44,45] For example, in the FRET process, non-radiative energy transfer as indicated in the Jablonski diagram (Fig. 2) occurs from the excited donor fluorophore to the nearby

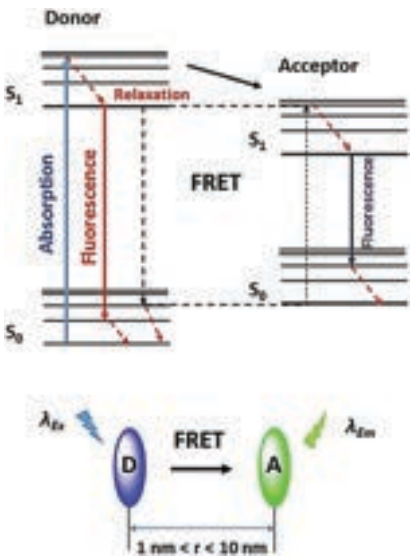

Fig. 2 The Jablonski diagram and illustration of the Förster Resonance Energy Transfer (FRET) process.

optical energy receptor. This phenomenon results in the quenching effect of the donor fluorescence, and consequently lights up the receptor molecules. Notably, the FRET efficiency is closely determined by two factors: a closer distance between the donor and acceptor (1–10 nm), and a greater spectral overlap between the donor emission and acceptor absorption spectra.[46,47] Based on this mechanism, a variety of activatable probes towards different analytes have been developed in recent years. To achieve more precise sensing performance, there are also diverse molecular probes fabricated with both targeted and activatable capabilities. In this article, we mainly concentrate on the recent progress of functional small-molecule probes, responsive principles and representative applications in the bacterial imaging field, which are discussed in detail below.

3. Small-molecule fluorescent probes for specific bacterial labeling and infection detection

In terms of the main difference of bacterial surface components in which the cell envelope of the Gram-negative (G^-) strains features as a double-membrane arrangement with almost exclusively lipopolysaccharides (LPS) on the outer membranes and different types of phospholipids in the inner membranes. In contrast, the single membrane of G^+ bacteria mainly contains teichoic acids and various phospholipids (Fig. 3).[48,49] Typically, the chemistry design in these probe molecules mainly relies on the conjugation of bacteria-targeted ligands, including positively charged chemical structures/peptides, bacteria affinitive carbohydrates, metabolizable precursors (*e.g.*, sugar or amino acids) and antibiotic derivatives. These targeted ligands mostly interact with the bacterial surface components, like polysaccharides, lipids and proteins, thus exerting specific labeling for bacterial detection. Furthermore, together with molecular design based on the bacterial surface targeted fluorescence labeling and sensing, there are some activatable probe molecules developed for bacterial imaging, especially under living conditions. In these typical studies, the fluorescent probes are mostly designed for specifically reacting with bacterial proteins, enzymes or pathological biomarkers (*e.g.*, radical species) and their fluorescence signal can only be activated upon external stimulation in a bacterial micro-environment, by which the minimal background interference and the greatly improved sensitivity and specificity can be easily achieved. Therefore, in accordance with the imaging purpose towards bacteria and infections, the different types of small molecule bacteria-specific imaging probes, their representative responsive chemistry principles and sensing applications are summarized in further details here.

3.1 Negatively charged bacterial cell wall targeted probes

Among the different bacteria-specific fluorescent probes, focusing on the negatively charged bacterial phospholipids on of the cell wall shows wide applicability to sense both Gram-positive and Gram-negative bacterial pathogens, but with less electrostatic interaction towards the membrane of healthy mammalian cells.[21,50,51] One of

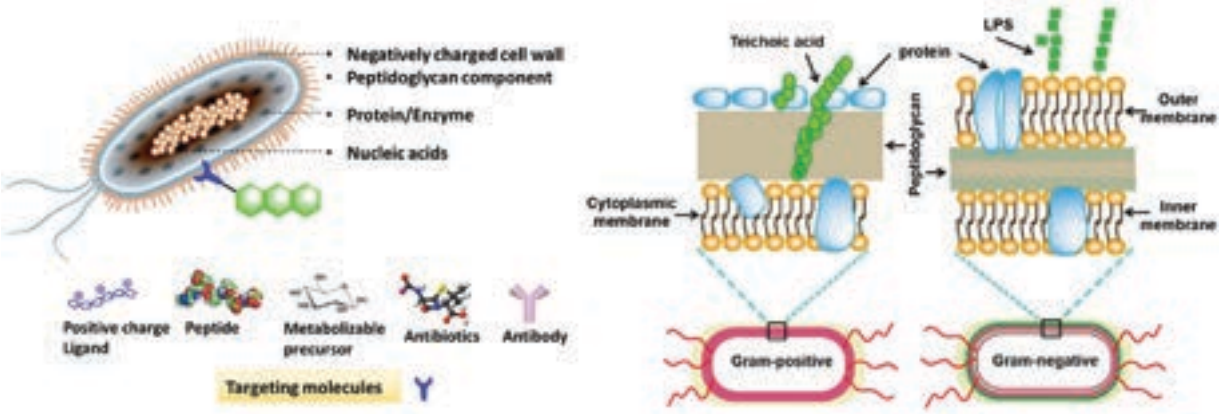

Fig. 3 Illustration of targeted probe molecules used for bacteria-specific imaging and bacterial cell membrane structures. Copyright 2020 Elsevier B.V.

the most commonly used positively charged chemical ligands is the metal complex zinc(II)-dipicolylamine (Zn-DPA), which has a high affinity for the negatively charged bacterial surface, thereby facilitating conjugation to several types of fluorophore labels (Fig. 4a).

For example, Smith *et al.* first reported a series of fluorescent probes with Zn-PDA as the bacterial recognition group.[52] Among them, the dansyl-based probe **1** demonstrates the capability to selectively associate with the cell membrane of *E. coli*, *P. aeruginosa* and *S. aureus* (Fig. 4b). The same group further developed a deep-red fluorescent pentamethine carbocyanine-based probe **2**, Cy5-like fluorophore with Zn-DPA covalent attachment for bacterial labeling.[53] As a superior substitute for Cy-5 fluorophores, Smith *et al.* also installed Zn-DPA on squaraine rotaxanes (**3**). Such kind of near-infrared probes hold the potential for fluorescent imaging of G^+ and G^- bacteria both *in vitro* and *in vivo* (Fig. 4c).[54,55] Another representative Zn-DPA tagged molecular probe **4** was designed on the basis of near-infrared Cy7 skeleton that has an emission wavelength at about 810 nm, which shows the feasibility for sensitive bacterial imaging in living mice (Fig. 4d).[56]

Other than Zn-DPA, quaternary ammonium derivatives can also act as positively charged ligands for effective bacterial targeting and inactivation (Fig. 5a).[57–59] Zhou *et al.* designed quaternary

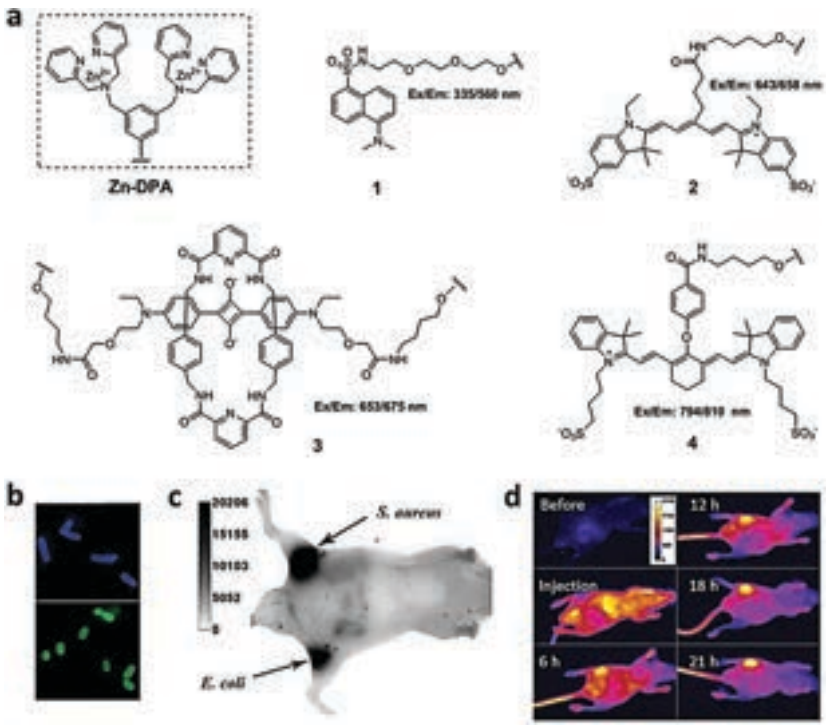

Fig. 4 Representative zinc(II)-dipicolylamine (Zn-DPA) linked molecular probes for bacterial labeling. (a) Chemical structures of Zn-DPA tagged probe molecules. (b) Optical image of *E. coli* stained with probe **1**. Copyright 2006 The Royal Society of Chemistry. (c) Optical image of a live mouse with subcutaneous injections of *S. aureus* and *E. coli* that were pre-labeled with probe **3**. Copyright 2007 Wiley-VCH. (d) Optical images of a mouse with *S. aureus* infection in the left rear thigh muscle with probe **4**. Copyright 2006 American Chemical Society.

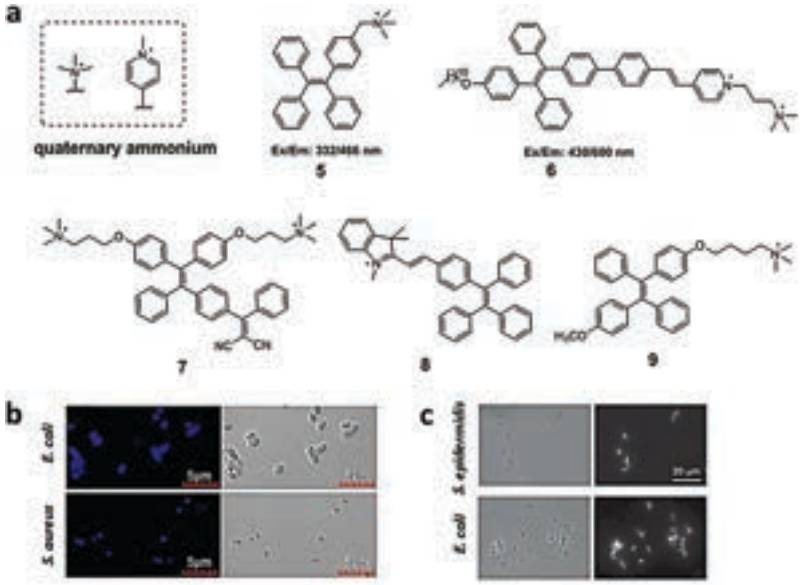

Fig. 5 Representative quaternary ammonium tagged molecular probes for bacterial labeling. (a) Chemical structures of quaternary ammonium-tagged AIE molecular probes. (b) Optical image of *E. coli* and *S. aureus* labeled with **5**. Copyright 2017 American Chemical Society. (c) Optical image of *E. coli* and *S. epidermidis* labeled with probe **6** through a wash-free method. Copyright 2015 Wiley-VCH.

ammonium linked tetraphenylethenes (TPEs) **5**, which were able to stain different kinds of bacteria through the concept of aggregation-induced emission (AIE) (Fig. 5b).[60–62] This type of probes was further fabricated to an efficient artificial tongue with fluorescent sensor arrays for bacteria discrimination. Tang *et al.* developed another AIE probe **6** that could be used for wash-free fluorescent imaging of bacteria (Fig. 5c), and subsequently used for bacteria susceptibility evaluation and high-throughput antibiotic screening.[63] Jiang *et al.* successfully developed a fluorescent sensor array composed of different color emissive AIE probes (**7–9**), which can be used for differentiating eight kinds of bacteria including normal and multi-drug resistant bacteria in water.[64] Additionally, Xing *et al.* reported that the positively charged peptide derivatives also feasible for effective bacterial targeting and inactivation.[65,66] As indicated, these positively charged probe molecules show the promising specificity and can realize direct bacteria surface labeling through the electrostatic and hydrophobic interactions.

3.2 Antibiotics-inspired molecular probes

Antibiotics have been widely used as bacterial targeting moieties for specific infection imaging, mostly owing to their high affinity towards bacterial cell membrane peptidoglycan structures and intra-bacterial proteins or enzymes.[21,67] Several antibiotics such as polymyxin B, ramoplanin, penicillin G and vancomycin were reported for bacterial imaging with fluorescent dyes conjugation (Fig. 6a).

Among them, Scocchi *et al.* applied Bodipy-labeled polymyxin B **10**, and Edelhauser *et al.* used Bodipy-labeled polymyxin B, ramoplanin **11**, penicillin G **12** for bacterial staining and further used as a control to evaluate the cell-penetrating properties of antimicrobial peptides (Fig. 6b).[68,69] Wang and Chen also explored Cy3-labeled polymyxin B for specific fluorescent imaging of G^- bacteria in living microbiotas.[70] Walker *et al.* prepared fluorescein- or bodipy-labeled vancomycin and ramoplanin, respectively, for living bacteria imaging and investigated the mechanisms of peptidoglycan biosynthesis (Fig. 6c).[71] Notably, extensive studies validated that vancomycin is a potent affinity ligand towards the G^+ bacteria cell wall, owing to the specific affinity with the D-Ala–D-Ala moiety in the G^+ bacterial surface peptidoglycan component through multiple hydrogen bonds interactions.[72,73] In this regard, Xing *et al.* designed a divalent vancomycin–porphyrin conjugate **14** to image bacteria, which exhibits a relatively higher binding affinity to bacterial surface than that of mono-vancomycin modified probes.[74–76] Liu *et al.* reported a red fluorescent vancomycin probe **15** that demonstrated high affinity to Gram-positive pathogens including vancomycin-resistant *Enterococcus* (VRE) strains. Based on aggregation-induced emission (AIE) mechanism, such light-up probe could realize direct naked-eye visualization of G^+ bacteria strains with the help of graphene oxide (GO) to eliminate the background signal.[77] Chen *et al.* synthesized Cy3-tagged vancomycin **16** for the cell-selective labeling of bacteria in the mouse gut microbiota (Fig. 6d).[78] Wang *et al.* recently synthesized Cy5.5-conjugated vancomycin for selectively targeted bacterial imaging and flow cytometry analysis.[79] Dam *et al.* explored the use of fluorescently labeled Cy7-vancomycin **17** to specifically target and detect *in vivo* infections caused by G^+ bacteria. In addition, Liu and Xu *et al.* presented a rhodamine-modified vancomycin probe **18** for bacterial imaging, and the peptide derivative conjugation further facilitates the formation of self-assemblies on the bacterial surface for effective infection sensing in a murine model (Fig. 6e).[80]

3.3 Carbohydrate-derived molecular probes

Carbohydrates are essential chemical components for bacterial membrane formation, replication and energy demand.[21]

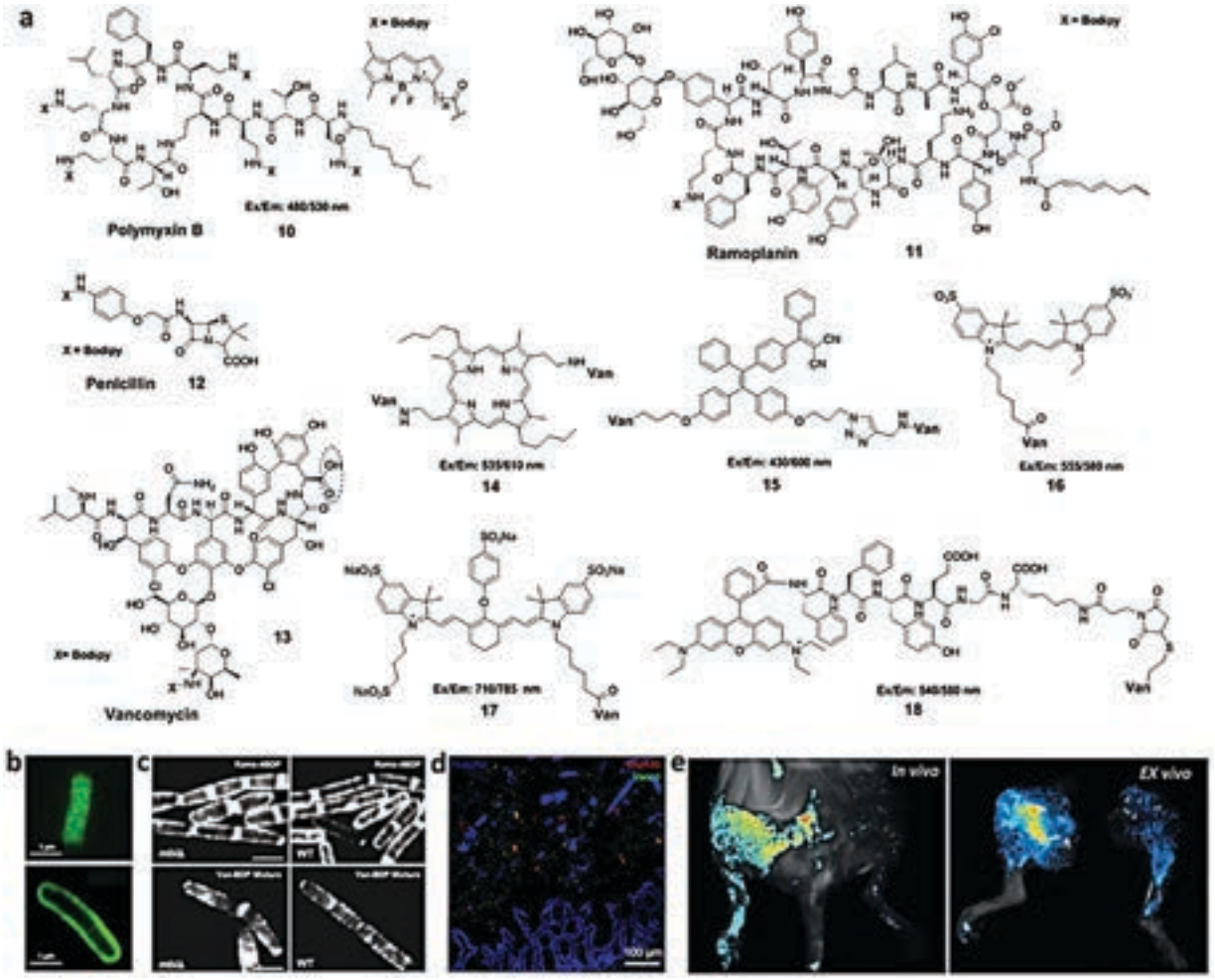

Fig. 6 Representative antibiotics-inspired fluorescent molecular probes for bacterial labeling and infection detection. (a) Chemical structures of fluorescently labeled antibiotics. (b) Fluorescent image of *E. coli* cells exposed to Bodipy-labeled peptides (upper) and probe **10** (lower). Copyright 2009 American Society for Microbiology. (c) Optical images of strains stained with **11** (upper) and **12** (lower), respectively. Scale bars, 2 μm. Copyright 2006 The National Academy of Sciences of the USA. (d) Fluorescence imaging of G^- and G^+ microbiotas (labeled with **16**) in the mouse gut in intestinal sections from mice. Copyright 2017 American Chemical Society. (e) *In vivo* and *ex vivo* imaging of infected mouse with methicillin-resistant *Staphylococcus aureus* (MRSA)-induced myositis in the left hind leg and *E. coli*-induced myositis in the right hind leg 2 h after i.v. injection of probe **18**. Copyright 2017 Wiley-VCH.

Accordingly, the bacterial cell membrane interacts with carbohydrates, which is proposed to be a useful strategy for bacterial targeting, recognition and/or active incorporation. So far, some carbohydrate-modified fluorescent molecules have been developed for bacterial imaging and infection sensing *in vivo* (Fig. 7a). As a non-mammalian disaccharide, trehalose is incorporated in the cell membrane of mycobacteria by trehalose mycolyltransesterase. As thus, Davis *et al.* designed a fluorescein-containing trehalose probe **19** (FITC-trehalose), which enabled the selective fluorescent labeling and imaging of *M. tuberculosis*, as well as sensitive detection of bacteria infected mammalian macrophages (Fig. 7b).[81] Differently, Gambhir *et al.* designed a maltotriose-conjugated fluorescent derivative **20**, which is not only capable of specifically labeling G^+ and G^- bacterial strains *in vitro* (Fig. 7c), but also enabling *in vivo* fluorescence and photoacoustic imaging of infection, visualization of the effectiveness of antibiotic treatment in *E. coli*-induced myositis as well in a clinically relevant *S. aureus* wound infection murine model.[82] Murthy *et al.* presented the maltodextrin-based fluorescent probe **21** that has high specificity and sensitivity for bacterial imaging and infection sensing in living mice (Fig. 7d).[83] In addition, Titz *et al.* used fluoresceine-derivative galactosides for specific staining of bacterial biofilms through the covalent interaction with a carbohydrate binding site.[84]

3.4 Metabolizable precursor-inspired molecular probes

The use of metabolic oligosaccharide engineering to bacterial cells glycan paves a new road to deliver imaging agents and therapeutics.[85] In this aspect, Dube *et al.* have already systematically described the mechanisms and applications in a previous work.[86] In this section, we just focus on the representative bacterial metabolic labeling studies and some recent advances in this field. Based on basic metabolic differences between

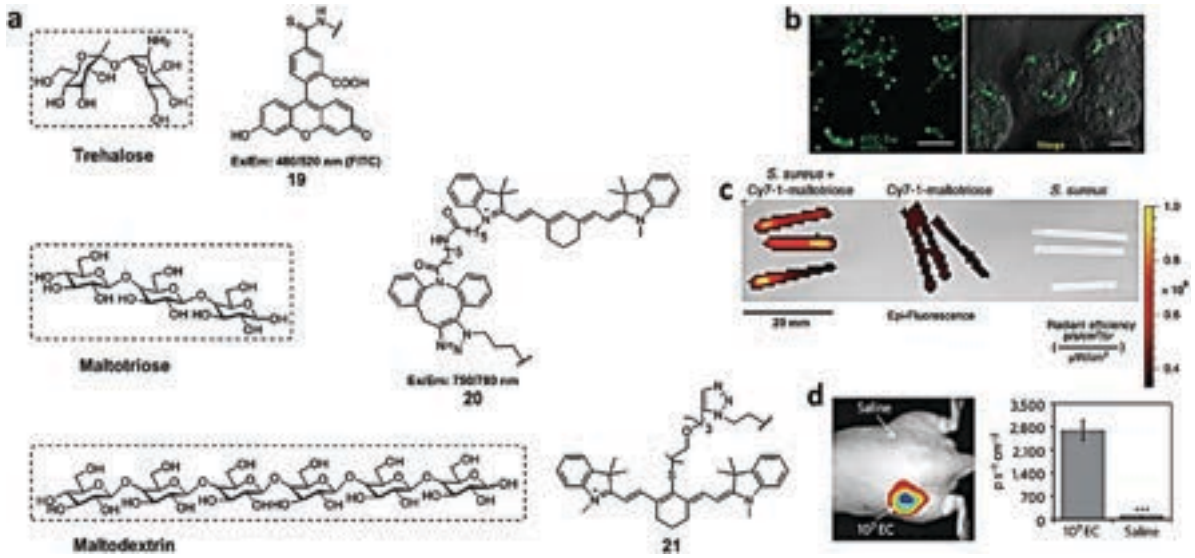

Fig. 7 Representative carbohydrate-based molecular probes for bacterial labeling and infection detection. (a) Chemical structures of carbohydrate-based molecular probes. (b) Fluorescent images of FITC-trehalose probe **19**-labeled bacteria and its infection in macrophages. Copyright 2011 Nature Publishing Group. (c) Fluorescent signals in catheters incubated with *S. aureus* and followed by probe **20** (left) incubation compared to sterile catheters incubated with **20** (middle). Copyright 2020 Nature Publishing Group. (d) Fluorescence image and quantification of fluorescence intensity of a rat with *E. coli* injection *in vivo* post probe **21** administration. Copyright 2011 Nature Publishing Group.

bacteria and mammalian cells, some unnatural metabolic precursors are available for bacterial uptake and incorporation into the peptidoglycan or lipopolysaccharide (LPS) structures. In particular, bioorthogonal (or click) reactions including copper(I)-catalyzed alkyne–azide cycloaddition (CuAAC) and copper-free cyclooctyne–azide cycloaddition (Fig. 8a) play an essential role in the covalently labeling of the metabolized bacteria.[87,88] In general, there are two typical strategies of the metabolic labeling process: one is the two-step approach through the successive bacterial metabolic incorporation and fluorescent labeling procedures; another is the one-step labeling that the metabolizable precursors are directly linked with the fluorophores (Fig. 8b).

Among different metabolically biorthogonal precursors, azide- and alkyne-bearing D-amino acids (*e.g.*, D-Ala, **22** and **23** in Fig. 9a) results in the specific labeling of newly synthesized peptidoglycan on the bacterial surface.[89] Besides, the use of bioorthogonally modified dipeptide probes (D-Ala–D-Ala, **24**) is

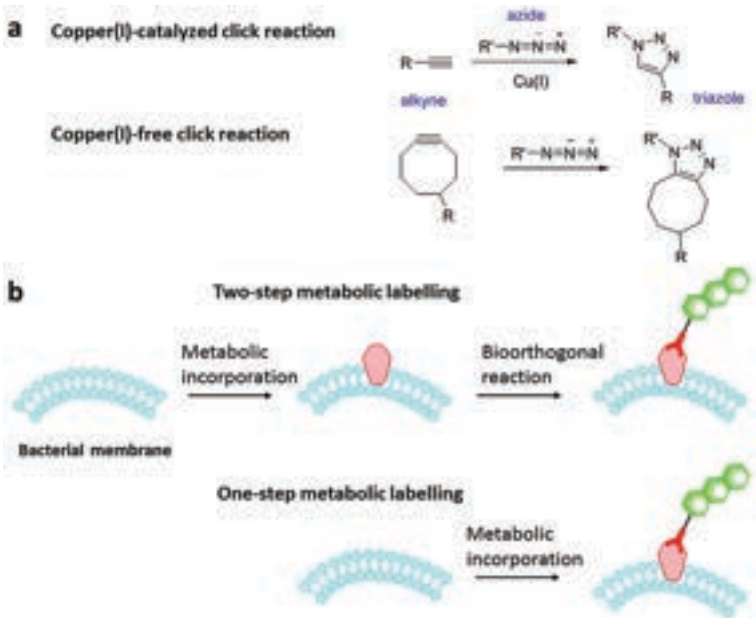

Fig. 8 (a) Typical click reactions for the covalent elaboration. (b) Illustration of two different metabolic labeling strategies.

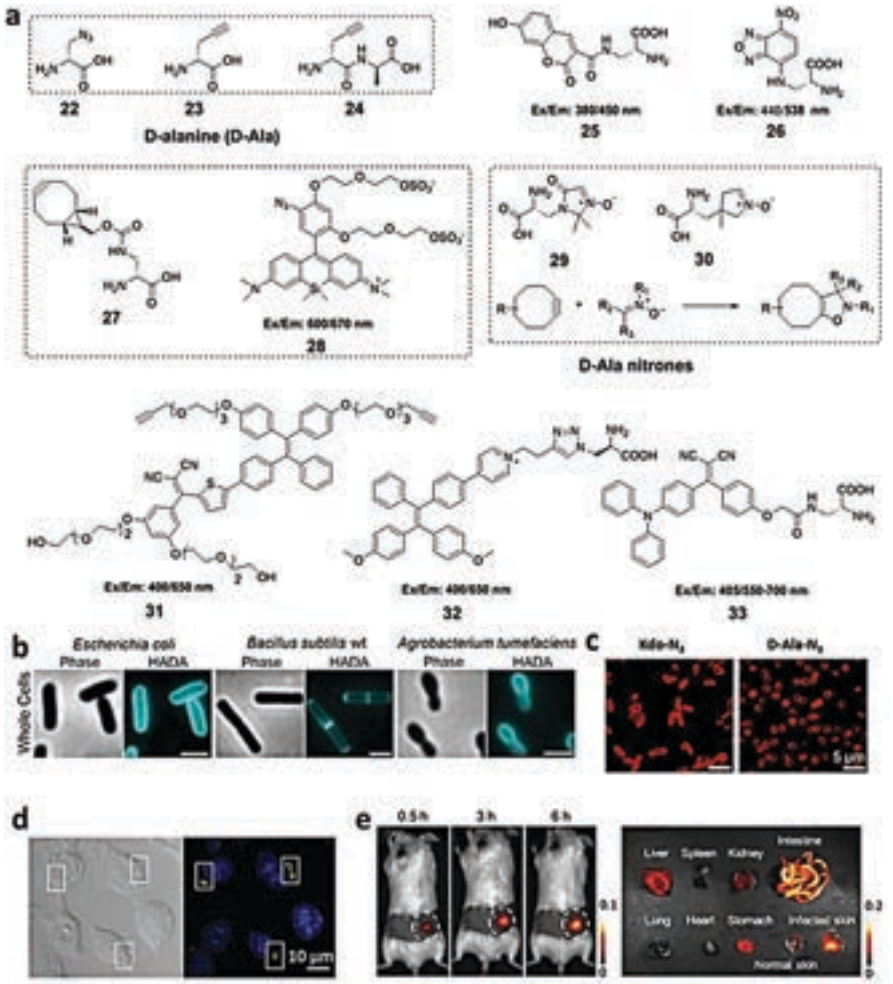

Fig. 9 Representative D-Ala based molecular probes for bacterial metabolic labeling and infection detection. (a) Chemical structures of D-Ala based bioorthogonal molecular probes. (b) Fluorescent labeling of bacterial strains with metabolic probe **25**. Copyright 2012 Wiley-VCH. (c) Cell-selective labeling of G^- bacteria (*E. coli*) and G^+ bacteria (*S. aureus*) with different metabolizable precursors pretreatment and probe **31** addition. Copyright 2019 American Chemical Society. (d) Fluorescent imaging and labeling of intracellular bacteria. Copyright 2013 Nature Publishing Group. (e) *In vivo* metabolic labeling-guided bacterial theranostics. Left: Time-dependent fluorescence images of bacteria-bearing mice after i.v. injection with **33**; right: *ex vivo* imaging of the probe biodistribution. Copyright 2020 The Royal Society of Chemistry.

also capable of metabolically labeling peptidoglycan in intracellular bacterial species (Fig. 9d).[90] Among these studies, VanNieuwenhze *et al.* prepared a serial of D-Ala functionalized fluorescent molecules (**25**, **26**) that allow for selective incorporation into the bacterial peptidoglycan cell wall (Fig. 9b).[91] Bertozzi *et al.* developed a copper(I)-free and NIR bioorthogonal system (**27** and **28**) for the visualization of a bacterial peptidoglycan without the need to wash away the unreacted probe.[92] Pezacki *et al.* further presented D-Ala derivatives bearing different endocyclic nitrones (**29** and **30**) that enable effective bacterial labeling through strain-promoted alkyne–nitrone cycloaddition (SPANC) reactions.[93] In particular, Liu *et al.* integrated metabolic labeling with the AIE molecules **31** and **32** for specific discrimination and elimination of bacterial pathogens *via* Kdo-N_3 and D-Ala-N_3 treatment (Fig. 9c).[94,95] Another bacterial metabolic probe **33** combining D-Ala and an AIE-photosensitizer has been reported by the same group, allowing for *in vivo* light- up imaging of bacterial infection and enhancing the efficiency of antibacterial therapy (Fig. 9e).[96]

Other than the unnatural amino acids, some bioorthogonally modified monosaccharides have also been used for bacterial metabolic labeling based on the specific incorporation into the peptidoglycan and lipopolysaccharides structures (Fig. 10a). For example, Vauzeilles *et al.* demonstrated that the Kdo analogue **34** can be metabolically assimilated and conjugated with bacterial LPS without the use of genetically modified bacteria, which shows potential applications in bacterial cell imaging (Fig. 10b), drug delivery, extraction and characterization of LPS.[97] Kasper *et al.* further applied such a technique with Kdo-N_3 and *N*-acetyl-galactosamine analogue **35** to image and track the path of labeled bacteria into specific intestinal system in the living murine host during health and disease (Fig. 10c).[98,99] Besides, Grimes *et al.* prepared *N*-acetyl-muramic acid derivatives **36**, **37** and investigated the installation of these

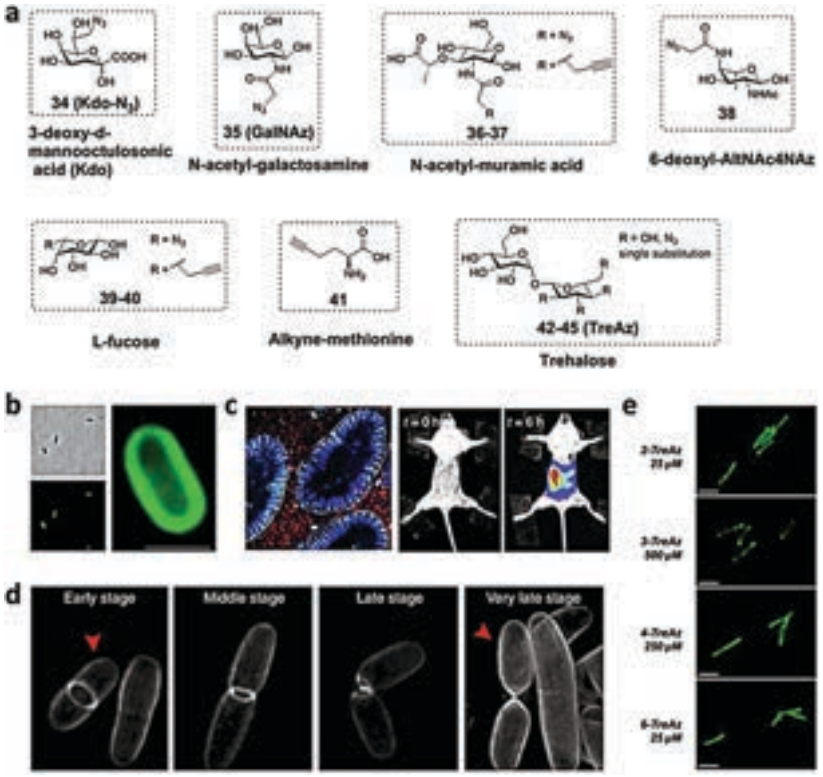

Fig. 10 (a) Chemical structures of other bioorthogonally modified molecules for specific bacterial metabolic labeling. (b) Fluorescent images of metabolically incorporated **34** in *E. coli* K12. Scale bar = 1 μm. Copyright 2012 Wiley-VCH. (c) Representative images from intravital two-photon microscopy of the small intestine after metabolic labeling of *B. fragilis* with TAMRA (left, blue represents Hoechst nuclear dye, scale bar, 10 μm) and hole-body imaging by IVIS after administration of Cy7-labeled *B. fragilis* in conventional SPF mice (right). Copyright 2015 Nature Publishing Group. (d) Fluorescent images of metabolically labeled *E. coli* at different cell division stages compiled from separate cell sample populations. Scale bar = 1 μm. Copyright 2016 Nature Publishing Group. (e) Fluorescence imaging of different TreAz-labeled bacteria. Scale bar = 5 μm. Copyright 2012 American Chemical Society.

building blocks into the backbone of a bacterial cell wall at different cell division stages (Fig. 10d).[100] Tanner *et al.* used the metabolic precursor 6-deoxy-*alt*-NAc_4NAz, **38** for specific bacterial engineering.[101] In another study, Dube *et al.* also demonstrated the feasibility for metabolic labeling of bacterial glycan and selective delivery of immune stimulants.[102] Wu *et al.* reported that both modified L-fucose derivatives **39–40** enable the selective labeling and detection of fucosylated glycoproteins from cultured bacteria.[103] In addition, Salje *et al.* developed a clickable methionine **41** as a universal probe for the labeling and fluorescent imaging of intracellular bacteria.[104] Bertozzi *et al.* revealed that azide-substituted trehalose derivatives **42–45** are processed by mycobacteria and incorporated into their glycolipids (Fig. 10e), which provides the potential for studying the trehalome during mycobacterial infection in host cells and model organisms.[105]

3.5 Bacterial enzyme-responsive molecular probes

Despite the great promise of cell-targeted strategies for bacterial fluorescence imaging, such a technique is still limited by the non-specificity and high-background signal interference. To overcome these issues, the design of stimuli-responsive molecular probe towards bacterial proteins, enzymes, and bioactive species provides an alternative approach for sensitive bacterial imaging and sensing.[20,106] In this section, we specifically highlight the representative principles and the examples using enzyme-responsive small-molecule probes for fluorescent imaging of bacteria, and resistant bacterial infection detection.

3.5.1 β-Lactamase-responsive fluorescent probes. β-Lactamases (Blas) are a family of bacterial enzymes produced as a means of self-defense that cleave β-lactam antibiotics, *e.g.*, penicillin, cephalosporin and carbapenem, rendering antibiotic resistance of bacterial infection towards the clinical therapy.[107–109] The pioneering research towards Blas enzymes optical imaging was introduced by Tsien, Rao and colleagues using FRET or PET-based (also called reporter enzyme fluorescence, REF) fluorescent antibiotic substrates **46–48** (Fig. 11),[110–112] of which the cephalosporin moiety in the fluorescent systems acts as a chemical linker with the structure modified by the fluorophores. The bacterial resistant enzyme response can cleave the β-lactam ring in antibiotic linkage and destroy the energy or electron transfer process within the structures, therefore amplifying the fluorescence for specific imaging applications. So far, the sensing principle, optimization and applications of Blas responsive reporters have been well established and relevant studies have been summarized in previous works.[109,113] Thus, in this

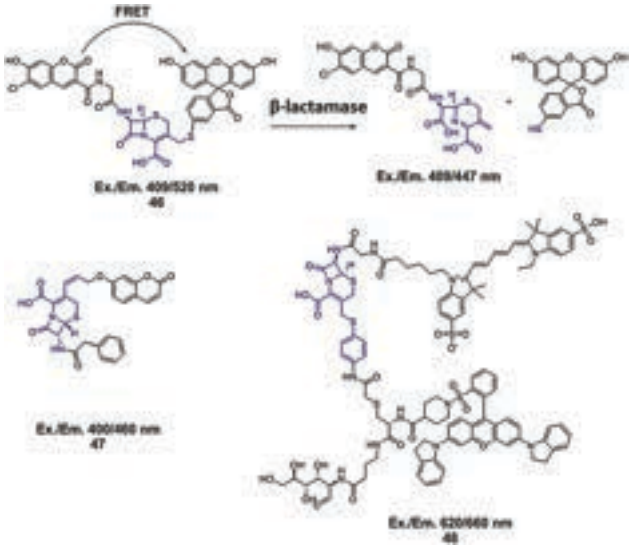

Fig. 11 Chemical structures of previously reported β-lactamase-sensitive fluorescent molecules for antibiotic resistant bacterial imaging.

article, we only focus on the latest advances based on literature reported over the last decade.

In recent years, some newly designed Blas probes have been designed for more specific and sensitive sensing of antibiotic resistant bacteria, as well infection detection in living mice (Fig. 12a). Among them, Cirillo *et al.* developed a NIR fluorogenic substrate **49** for β-lactamase-expressed bacterial analysis, which also allows for real-time imaging of pulmonary infections and rapid quantification of resistant bacteria in living animals (Fig. 12b).[114] Li and co-workers synthesized a ratiometric fluorescent probe **50** for quick and selective screening of resistant microbes towards methoxyimino cephalosporins.[115] With the aim to perform the clinical diagnosis of tuberculosis, Rao *et al.* reported BlaC-specific fluorogenic substrates (**51**) for *Mycobacterium tuberculosis* (Mtb) detection. This platform also provides the opportunity for the accurate and rapid sensing of very low numbers of Mtb for the clinical diagnosis of tuberculosis in sputum and other specimens.[116] The same group further modified a BlaC-specific probe with a bacterial targeting ligand trehalose (**52**), offering a versatile tool for tracking Mtb in both pre- and post-phagocytosis, and elucidating the fundamental pathophysiological processes related to the mycomembrane (Fig. 12c).[117] Besides, Xing *et al.* creatively designed a β-lactamase-induced covalent labeling strategy (**53–55**) for the quantitative and sensitive analysis of the resistant bacterial population, as well as direct observation of bacterial enzyme activity in resistant pathogenic species at the single-cell level (Fig. 12d).[118–120] Another study by Xing *et al.* reported that the enzyme-responsive reporter molecule **56** can specifically recognize AmpC β-lactamase in drug-resistant bacteria. Briefly, a typical tetraphenylethylene (TPE) moiety was chosen to link with the cephalosporin structure. The AmpC enzyme reaction triggered the release of TPE, which exhibited AIE-based fluorescence enhancement and thus enabled the selective localization of pathogenic biofilms (Fig. 12e).[121] Additionally, Murthy *et al.* presented a cephalosporin–chemiluminescent substrate conjugated probe **57** for the first time to detect β-lactamase activity *via* chemiluminescence with good sensitivity.[122]

There are also some recent studies focusing on the fluorescent sensing of carbapenemase-expressing resistant bacterial strains (Fig. 13). For example, Leonard *et al.* introduced a fluorescent carbapenem **58** for specific binding and labeling of penicillin-binding proteins and β-lactamases. However, such a probe is not capable of enzyme activity screening.[123] In another study, Rao *et al.* successfully designed a fluorogenic probe **59** for the sensitive detection of carbapenemases, including metallo-β-lactamases in active carbapenem-resistant Enterobacteriaceae (CRE) pathogens based on stereochemically modified cephalosporin.[124] Xie *et al.* further developed a carbapenem-based fluorogenic probe **60** with an unprecedented enamine-Bodipy switch for the detection of carbapenemase activity and efficient imaging of clinically important carbapenemase-producing organisms (CPOs), showing the potential for rapid detection of antibiotic resistance and timely diagnosis of CPO infections.[125] Contemporaneously, Rao *et al.* reported a caging strategy based probe **61** for the fluorescent detection of metallo-carbapenemase-expressing bacteria.[126] Shabat *et al.* also described a carbapenemase-sensitive chemiluminescent probe **62**, which has the potential for future diagnostic assays, and the rapid and accurate detection of carbapenemase-resistant bacteria.[127]

3.5.2 Nitroreductase and other enzyme-responsive molecular probes. In addition to the aforementioned bacteria-resistant enzymes-responsive fluorogenic probes, some other bacterial enzymes-sensitive molecular probes have been recognized as effective tools for bacterial imaging and infection detection. In particular, nitroreductases existed mostly in bacteria with nicotinamide adenine dinucleotide (NADH) or nicotinamide adenine dinucleotide phosphate (NADPH) as a cofactor that have been used for metabolizing diverse nitroaromatic and nitro heterocyclic compounds, which have drawn great attention in the area of biomedicine, environmental, and human impacts.[128,129]

Among different bacterial nitroreductase imaging studies (Fig. 14a), Moernor *et al.* synthesized a nitro-aryl fluorogen **63** that can be used for specific localization of the enzyme on the single-molecule level in *B. subtilis* (Fig. 14b).[130] Tangney *et al.* used a commercially available nitroreductase probe **CytoCy5S** for both *in vitro* and *in vivo* optically bacterial imaging and infection monitoring.[131] Hu *et al.* reported a turn-on fluorescent probe **64** for the selective sensing of nitroreductase activity and initially applied in rapid, real-time detection and identification of ESKAPE pathogens.[132] The same group further designed and optimized nitroreductase fluorescent probes, of which the molecule **65** enables the ready penetration of the cell envelopes of bacteria, thus minimizing the background signal for the real-time intracellular enzymes imaging in live bacterial cells.[133] Besides, Lee *et al.* presented a resorufin-based fluorescent probe **66** for nitroreductase activity analysis using a wash-free process.[134] In particular, Ning *et al.* developed a nitroreductase-responsive fluorescent probe **67** for the rapid detection of Listeria *in vitro* and *in vivo* with high specificity and

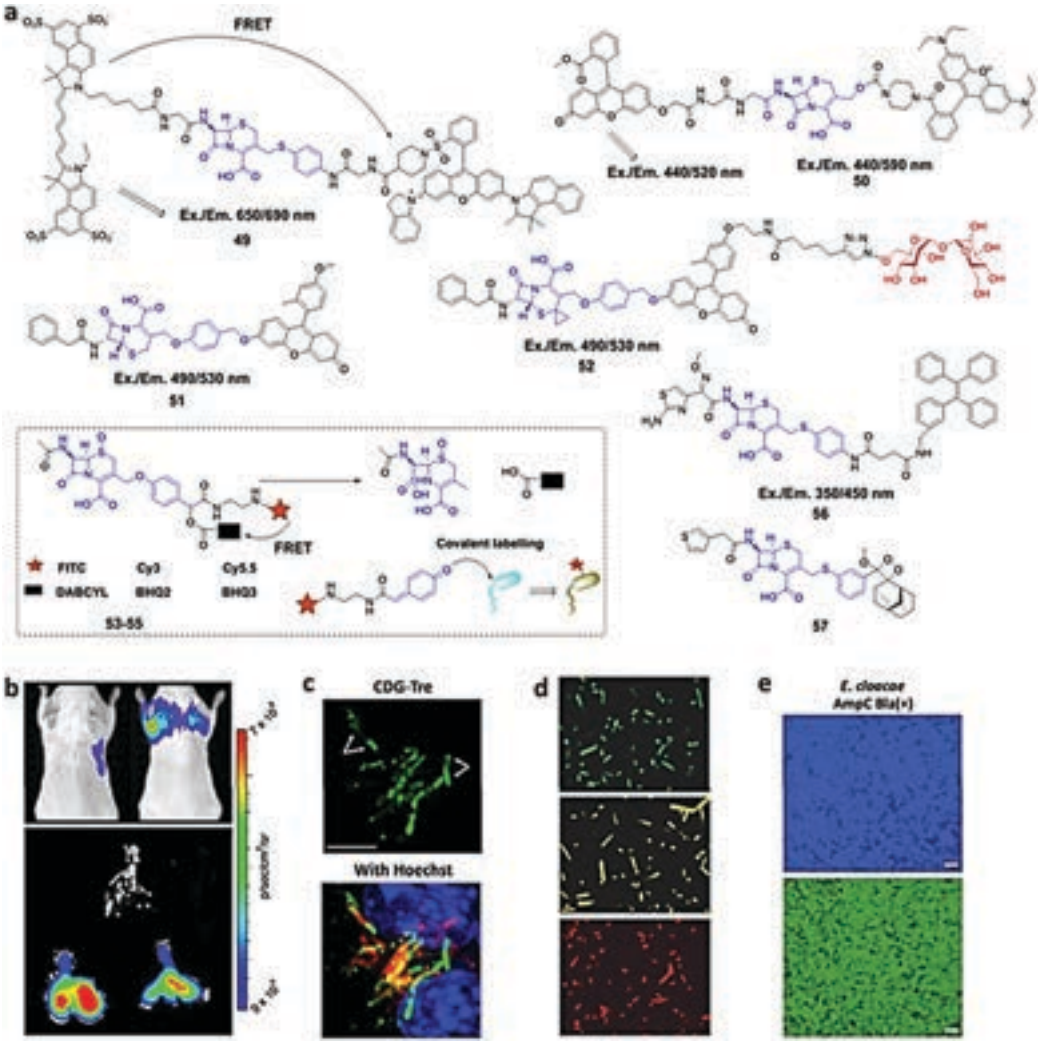

Fig. 12 Representative new β-lactamase-sensitive fluorescent molecular probes for bacterial imaging and infection detection. (a) Chemical structures of β-lactamase-responsive probes. (b) Fluorescent detection of bacillus Calmette–Guérin and *Mycobacterium tuberculosis* (Mtb) after pulmonary infection of mice with probe **49**. Copyright 2010 The National Academy of Sciences of the USA. (c) Fluorescent imaging of live bacterial cells within macrophage phagosomes with probe **52**. Copyright 2020 American Chemical Society. (d) Fluorescent imaging of penicillin resistant bacterial with probes **53–55**, respectively. Copyright 2013 Wiley-VCH. (e) Fluorescent imaging of penicillin resistant *E. cloacae* formed biofilms with probe **56**. (Green: bacterial GFP; Blue: probe response) Copyright 2017 The Royal Society of Chemistry.

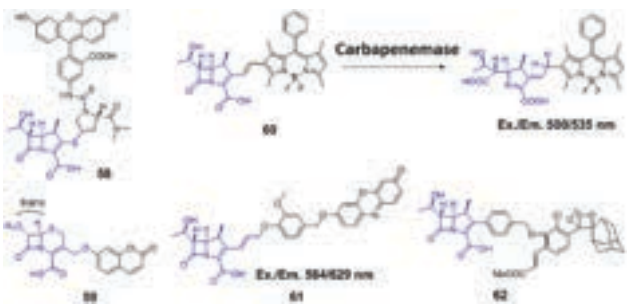

Fig. 13 Chemical structures of representative carbapenemase-sensitive fluorescent molecular probes for resistant bacterial detection.

sensitivity, which has a potential for bacterial infection diagnosis and antibiotics screening (Fig. 14c).[135] Another study by Hu *et al.* applied a Cy7-based NIR nitroreductase probe for rapid differentiation between bacterial infections and cancers (Fig. 14d).[136] Recently, Nazar and Hu *et al.* further developed a turn-on lanthanide luminescent probe **68** for time-gated detection of nitroreductase in live bacteria (Fig. 14e). This type of probe concept is promising for future analytical applications in medical diagnostics.[137]

Other than nitroreductase-sensitive probes for bacterial imaging, Groundwater *et al.* introduced a fluorogenic self-immolative substrate **69** that can be specifically hydrolysed by β-alanyl aminopeptidase, allowing for quick and efficient identification of pathogenic bacteria.[138] Cui *et al.* designed a γ-glutamyl transferase sensitive probe **70.** Such a NIR fluorescent molecule enables the visualization of γ-glutamyl transferase-expressing bacteria in murine stomach and intestines.[139] In addition, Hu *et al.* developed a FRET probe **71** for selective

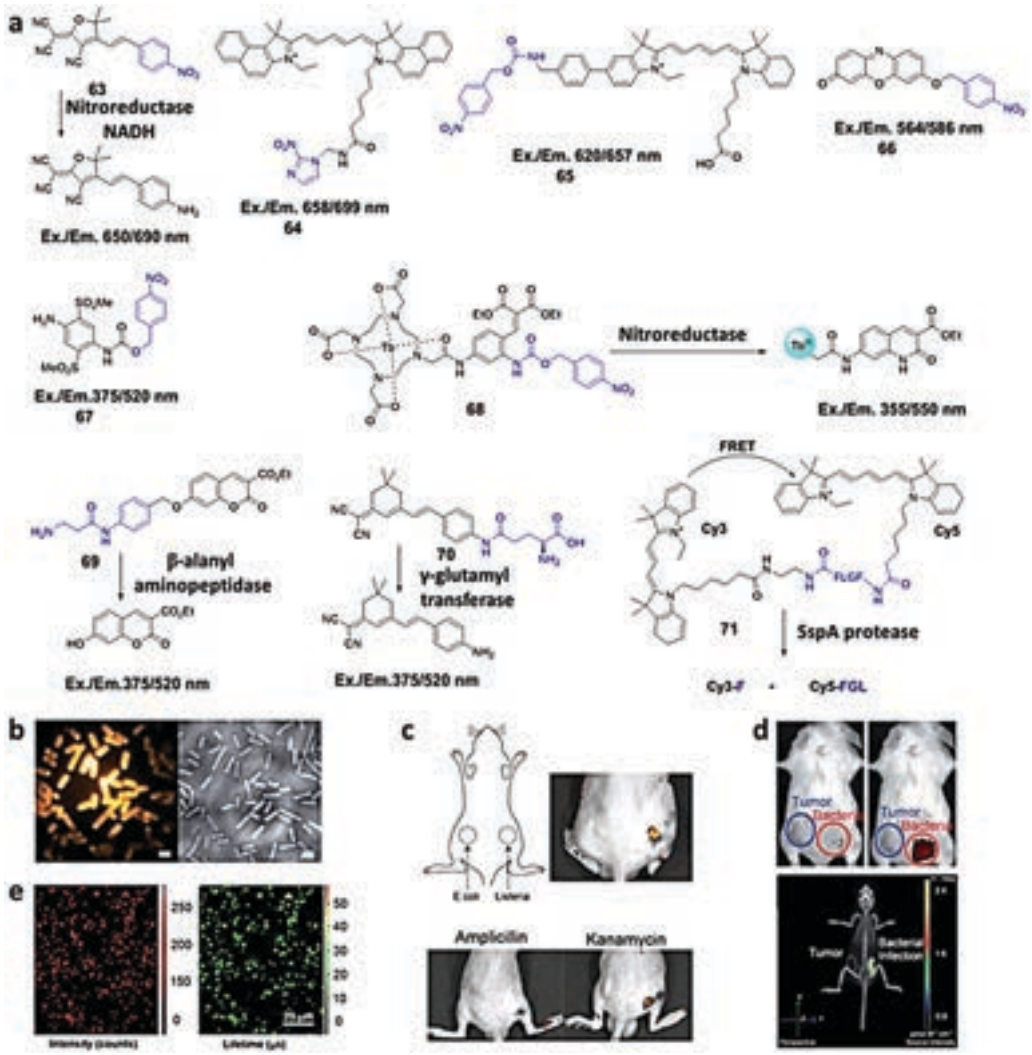

Fig. 14 Representative nitroreductase and other enzyme-sensitive fluorescent molecular probes for bacterial imaging and infection detection. (a) Chemical structures and responsive principles of the molecular probes. (b) Fluorescent image of *B. subtilis* incubated with probe **63**. Copyright 2013 The Royal Society of Chemistry. (c) *In vivo* fluorescent imaging of bacteria infected mice and antibiotics treatment with probe **67**. Copyright 2019 Elsevier B.V. (d) Whole-mouse optical imaging of tumor-bearing (blue circle) and *S. aureus* infected (red circle) mice before and post i.v. injection of Cy7-based nitroreductase probe. Copyright 2020 The Royal Society of Chemistry. (e) Fluorescence lifetime imaging of live *E. coli* bacteria incubated with probe **68**. Copyright 2020 Wiley-VCH.

real-time and ratiometric imaging of endogenous extracellular protease SspA activity in live *S. aureus* cells.[140]

3.6 Bacterial infection pathogenesis-activatable molecular probes

During the bacterial infection, there are inflammatory responses, immune activations and lethal actions that cause the pathogenesis of infectious diseases.[141] Among the various bioactive factors, the biological free radical species including reactive oxygen and nitrogen species (RONS, *e.g.* $O_2^{\bullet-}$, $^{\bullet}OH$, H_2O_2, NO, $ONOO^-$, ClO^-) are widely implicated to play essential roles in the progression of bacteria-induced inflammation and inducing intrinsic bactericidal effects.[142,143] There have been numerous ROS or RNS fluorescent imaging probes reported for diverse applications regarding the redox biology.[144,145] Notably, the specific imaging of live bacteria induced infection and related redox pathogenesis are rarely reported, because most of the studies established bacterial inflammation models by using chemo-stimulants, like phorbol 12-myristate 13-acetate (PMA), lipopolysaccharide (LPS), interferon-γ (IFN-γ), and others.[146–149] In this section, we specifically discuss the recent advances of redox-activatable small-molecule probes for fluorescent imaging of live bacterial infections.

As summarized in Fig. 15a, Yoon *et al.* designed rhodamine-based molecule probes (**72**) for the sensitive imaging of microbe-induced HOCl production *in vitro* and *in vivo* (Fig. 15b).[150–152] Ma *et al.* also developed a similar fluorescent probe with the mitochondrial-targeting ability, which allows for the confocal fluorescence imaging of HOCl generation in the mitochondria of macrophages during bacterial infection (Fig. 15c).[153] Tang *et al.* synthesized an AIE-active light-up molecular probe **73** for selective *in vivo* imaging of bacterial

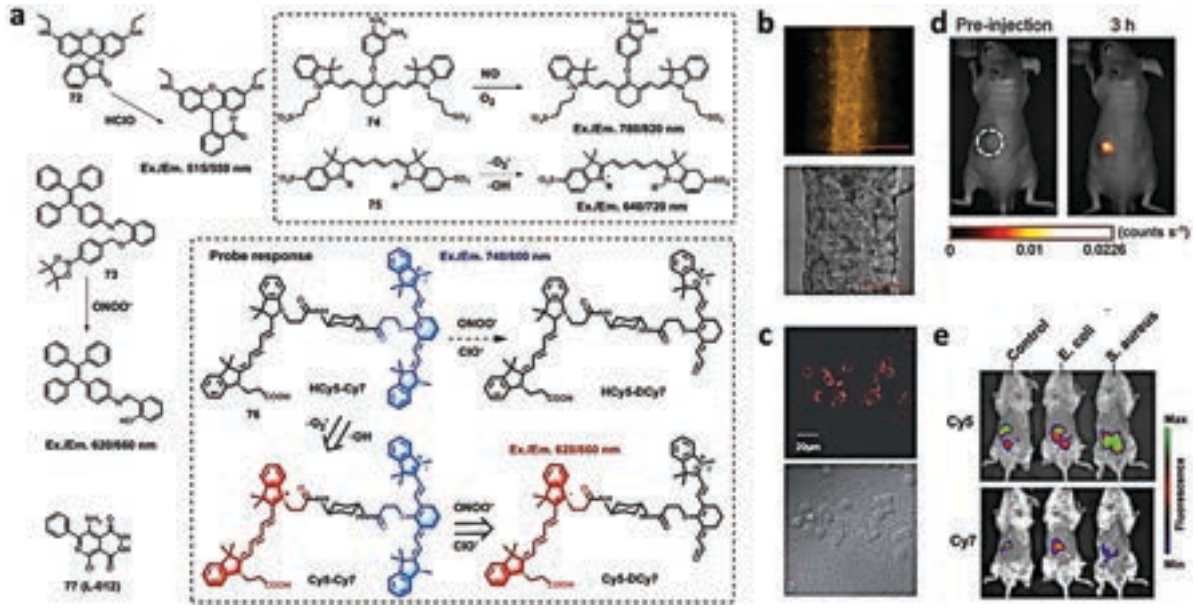

Fig. 15 Representative reactive oxygen and nitrogen species-sensitive molecular probes for fluorescent imaging of infection pathogenesis. (a) Chemical structures and responsive mechanisms of probe molecules. (b) Fluorescent imaging of HOCl production in the Drosophila intestine following Ecc15 bacterial infection with probe **72**. Scale bars: 50 μm. Copyright 2016 Nature Publishing Group. (c) Fluorescence images of RAW264.7 cells during *E. coli* infection. (d) *In vivo* fluorescence images of inflammation-bearing mice before and after i.v. injection of probe **73**. The white circles indicate the MRSA-infected region. Copyright 2016 Wiley-VCH. (e) *In vivo* fluorescence signals of mice infection post typical Gram-negative and Gram-positive bacterial infections with probe **76**. Copyright 2021 Wiley-VCH.

infection induced inflammation and efficient visualization of *in vivo* treatment efficacy of anti-inflammatory agents (Fig. 15d).[154] Besides, Garcia *et al.* exploited NIR fluorescence molecular probes combination (**74** and **75**) for minimally invasive and simultaneous imaging of inflammation, and infection associated with implanted polymer disks in mice.[155] Very recently, Xing *et al.* introduced a unique dual wavelength NIR cyanine-dyad molecular probe HCy5–Cy7 (**76**) for the simultaneous response to RONS variations both in bacteria-infected macrophages and living mice (Fig. 15e).[156] Briefly, HCy5–Cy7 specifically turned on 660 nm fluorescence *via* $O_2^{\bullet-}$ or $^{\bullet}OH$-mediated oxidation of reductive HCy5 to Cy5 moiety, while $ONOO^-$ or ClO^--induced Cy7 degradation led to the emission turn-off at 800 nm. Such a multispectral but reverse signal responses technique enables multiplex manifestation of *in situ* oxidative and nitrosative stress during the pathogenic and defensive processes of bacteria-induced infectious diseases. The final example uses a chemoluminescent probe 77 (L-012) that can be used for *in vivo* imaging of the overall RONS level and longitudinal progression in murine colitis.[157]

4. Summary and perspectives

In the past decades, small-molecule fluorescent probes have drawn much attention for their great merits in the biomedical imaging research. The diverse small molecule fluorophores with highly tunable emissions are particularly efficient and safe in chemical and biological analysis, owing to the high quantum yields and good biocompatibility. Furthermore, the rational design of fluorescent probes with complementary component modification facilitates more versatile and reliable signal response in the complex biological environment. Based on the above points, a variety of fluorescent probes towards different bioanalytes have been developed. In this feature article, we specifically summarize recent advances of small-molecule probes developed for fluorescent imaging of bacteria and infection, which covers different type of design strategies including electrostatic and hydrophobic interactions, metabolic oligosaccharide engineering, bacterial enzyme responses, and infection pathological activations.

Despite the remarkable progress in this field, there are several paramount challenges that need to be addressed towards the clinical translation. Basically, further refinements of small-molecule probes for effective diagnosis of bacterial infections include the following several aspects:

(1) Enhancing the stability and biosafety; most of the organic fluorophores, especially NIR fluorescent molecules with a large hydrophobic conjugate π–π system have the problems of photostability, such as agglomeration-induced fluorescence quenching, photobleaching and other photophysical changes, which makes them limit in the long-term tracking and high-resolution bioimaging in disease diagnostics.[158,159] Additionally, although there has been toxicity-related information available for the FDA-approved dyes like indocyanine green (ICG) and fluorescein, the biosafety of other types fluorescent molecules for clinical applications is less well known.[160,161]

(2) Improving the selectivity and sensitivity; despite the merits of targeted and activatable fluorescent probes designed for various bacteria and infections imaging, the specific recognition of the individual pathogen and profiling of its pathophysiological implications are still at the early stage. In particular, it is the lack of clinically usable small-molecule probes for quick and sensitive imaging of drug-resistant or even multi-drug resistant bacteria caused infections.

(3) Standardizing the operation and instrumentation; currently, the commercial *in vivo* imaging systems (IVIS) used for fluorescent imaging are mostly built for small animals, specialized instruments for human patients are very limited. Besides, unsatisfied reproducibility and robustness of optical imaging studies vary from different disease models in different research groups, it is a critical step for standardizing the instrumentations and operations that can enable comparative results, and meet the requirements for clinical usage.

(4) Encouraging the clinical practice and efforts: biomedical imaging is a highly multidisciplinary field, the successful accomplishment of clinical translations demands cross-continental cooperation and integration from academia and industry all over the world. At the same time, any trials and efforts towards clinical applications of such a technology deserve to be encouraged. Taken together, we envision that the small-molecule fluorescent probes for specific bacterial labeling and infection detection will have a bright future.

Conflicts of interest

The authors declare no conflict of interest.

Acknowledgements

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