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Bacterial membrane-induced disassembly of fluorescein aggregates enables selective imaging and killing of Gram-positive bacteria

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ABSTRACT

The escalating health threats posed by bacteria and the pressing issue of antibiotic resistance underscore the urgent need for selective detection technologies and antimicrobial reagents. Fluorescein's biocompatibility renders it the preferred fluorescent dye for in vivo imaging. This study reveals that compounds from the aliphatic chain-derived fluorescein series (**BMP-alkyl**) not only selectively stain and fluorescently image Gram-positive bacteria but also demonstrate the ability to rapidly eliminate them. The discovery of fluorescein's antibacterial properties is poised to have applications in antibiotic research due to its excellent biocompatibility. These amphiphilic **BMP-alkyl** compounds were easily synthesized through a one-step amidation process involving dicarboxyfluorescein and long-chain fatty amines. In aqueous solutions, the formed aggregates exhibited complete fluorescence quenching. Upon encountering the Gram-positive bacterial membrane, **BMP-alkyl** compounds underwent depolymerization, resulting in a single fluorescent molecule and the activation of fluorescence. Subsequently, as these amphiphilic fluoresceins continuously combined and entered the bacterial interior, the potential for bacterial membrane depolarization, membrane structure damage, and ultimately bacterial death ensued.

1. Introduction

Pathogenic bacterial infection seriously damages human health. Antibiotics were widely used as an effective means of treating bacterial infections, however, with the continuous evolution of pathogens and the overuse of antibiotics, antibiotic-resistant bacterial has caused a global public health crisis which become an urgent problem to be solved. [1,2] Bacterial diagnosis is crucial to alleviate this situation, as it enables timely guidance for medication and prevents the development of drug resistance in pathogenic bacteria. While Gram staining has traditionally served as the gold standard technique for bacterial classification, relying on the distinct physical properties of bacterial cell wall structures, its multi-step process, involving bacterial fixation, staining, decolorization, and washing, proves cumbersome. Importantly, this method lacks the ability for real-time bacterial detection, and the decolorization step often leads to inaccurate Gram determinations.[3]

Fluorescence imaging technology boasts a broad spectrum of applications in bacterial research and detection, thanks to its remarkable features such as high sensitivity, rapid response, and real-time observation capabilities.[4–6] In recent years, fluorescent probes designed for bacterial detection primarily target bacterial surfaces, cell walls, proteins, nucleic acids, and enzymes.[7–9] Notably, a simple and effective strategy involves selectively distinguishing between Gram-negative and Gram-positive bacteria based on differences in their bacterial surfaces. For example, naphthalimides modified with hydrophobic alkyl chains have demonstrated the capability to selectively detect Gram-positive bacteria through variations in bacterial membrane dispersion and depolymerization abilities.[10,11] Through the strategic combination of boronic acid groups with peptidoglycan on the surface of Gram-positive bacteria and the optimization of the physical properties of BODIPY molecules, successful detection of positive bacteria in mice has been achieved. [12,13] Currently, the existing probes capable of selectively distinguishing between bacterial types face a limitation in terms of sterilization. Therefore, finding a solution for the integration of bacterial diagnosis and treatment using these probes holds great significance.

In the pursuit of effectively eliminating bacteria without triggering drug resistance, various antimicrobial agents have been developed and

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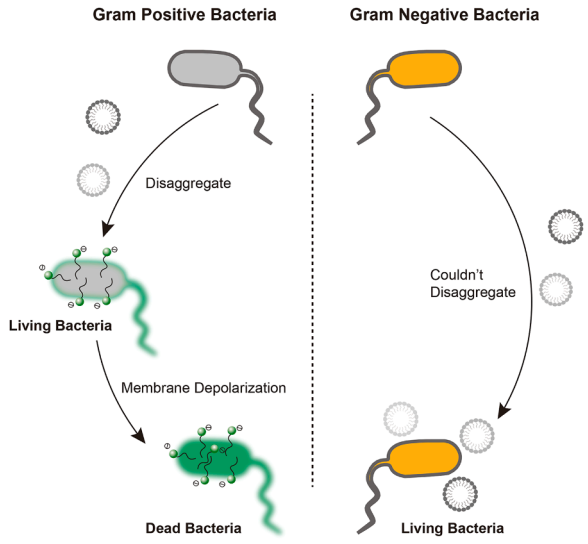
employed. These include fluorescent dyes, cationic antimicrobial polymers, antimicrobial peptides, photosensitizers, and other innovative compounds. [14–18] In this array of antimicrobial agents, fluorescent dyes emerge as particularly promising for the integration of bacterial diagnosis and treatment, providing an avenue for in-depth exploration of the mechanisms underlying bacterial elimination. However, it's crucial to highlight that while certain rhodamine dyes demonstrate noteworthy antimicrobial properties, their application in vivo is constrained by their inherent cytotoxicity, limiting their broader antimicrobial potential.[19–21] Fluorescein dyes possess high brightness and excellent biocompatibility, making them suitable for applications within the human body.[22,23] Despite these favorable characteristics, there has been limited reporting on the use of fluorescein as a bacterial imaging and antibacterial agent.

In this study, we report fluorescein derivatives designed for Gram-positive bacterial membrane-driven disassembly, aiming to facilitate screening, imaging, and targeted bacterial elimination in situ (Scheme 1). The aggregation of **BMP-alkyl**, induced by the hydrophobic alkyl chain group, effectively quenches fluorescence, providing a lower background signal. **BMP** stands for buffering membrane probe, indicating that the fluorescence quenching aggregates formed can consistently stain the cell membrane. Simultaneously, the negative charge of fluorescein prevents the amphiphilic **BMP-alkyl** from binding to Gram-negative bacteria due to electrostatic repulsion. Upon encountering Gram-positive bacteria, the aggregated **BMP-alkyl** is depolymerized, forming a single fluorescent unit on the bacterial membrane, activating the fluorescent signal. Subsequently, as fluorescein derivatives continue to transfer into the bacterial interior, the bacterial membrane potential decreases, and the bacterial membrane structure is compromised, ultimately achieving selective killing of Gram-positive bacteria. We systematically investigated the biological properties of amphiphilic fluorescein derivatives through various tests, including bacterial detection and imaging, membrane potential changes, and antibacterial efficacy, revealing their potential application in the integration of bacterial diagnosis and treatment.



Scheme 1. The schematic mechanism of **BMP-alkyl** for selectively imaging and killing Gram-positive bacteria. Aggregation induced by hydrophobic alkyl chain groups effectively quenches fluorescence. Upon encountering Gram-positive bacteria, aggregated **BMP-alkyl** depolymerizes, activating a fluorescent signal. It then penetrates the bacteria, decreasing membrane potential and damaging membrane structure, selectively killing Gram-positive bacteria. However, it does not affect Gram-negative bacteria in terms of labeling or killing.

2. Results and discussion

2.1. Aggregation-disaggregation regulates fluorescence switch

The five fluorescein derivatives in this paper feature a hydrophobic alkyl chain structure and a hydrophilic fluorescein, resulting in appropriate amphiphilicity and the ability to aggregate in aqueous solutions (Fig. 1a). The synthesis pathway for **BMP-alkyl** is outlined in Figure S1, and the chemical structures of all compounds were confirmed through nuclear magnetic resonance spectrometry and high-resolution mass spectrometry. The initial focus was on characterizing the photophysical properties of the five fluorescein derivatives. Notably, three of them (**BMP-14**, **BMP-16,** and **BMP-18**) exhibited minimal fluorescent signals in phosphate-buffered saline (PBS) due to the aggregation-caused quenching (ACQ) effect. Their quantum yields in PBS were measured as 0.06, 0.01, and 0.01, respectively. However, each of these derivatives displayed a substantial increase in fluorescence—4-fold, 23-fold and 28-fold, respectively—upon the addition of sodium dodecyl sulfate (SDS) (Figure S2). This enhancement is attributed to SDS dispersing the aggregates. And **BMP-alkyl** also responded to other surfactants, for example Tween-20 and DTAC (Figure S3-S5).

The fluorescence intensity of the other two fluorescein derivatives, **BMP-8** and **BMP-12**, exhibited high quantum yields of approximately 0.6, which slightly decreased after the addition of the surfactant SDS. Further analysis of the absorption spectra of fluorescein derivatives before and after adding SDS revealed that the molar extinction coefficients of the latter three fluorescein derivatives (**BMP-14**, **BMP-16**, and **BMP-18**) were less than half of those of the first two fluorescein derivatives (**BMP-8** and **BMP-12**). This indicates that the amphiphilic fluorescein derivatives (**BMP-8** and **BMP-12**) were unable to aggregate in aqueous solutions at low concentrations due to the short length of their alkyl chains. In previous studies,[24] it was observed that the structure of fluorescein undergoes changes with increasing acidity, leading to noticeable differences in its absorption spectrum. The absorption of **BMP-8** slightly decreased after the addition of surfactant SDS. Therefore, we speculate that the negative charge of the SDS surfactant induced a change in the parent structure of fluorescein, resulting in a decrease in absorbance and a reduction in fluorescence intensity. However, the absorption spectra remained largely unchanged.

Conversely, after the addition of surfactant SDS, the absorbance of **BMP-12** was halved, and the absorption wavelength experienced a blue shift, with two new absorption peaks appearing. This phenomenon may be attributed to **BMP-12** being able to insert into the micelles of the SDS surfactant, causing fluorescein to transition from the oxygen-negative to the phenolic hydroxyl form. This suggests that **BMP-12** could potentially insert into bacterial membranes, albeit with weak binding capacity. Given the superior aggregation and fluorescence quenching behaviors observed in **BMP-14**, **BMP-16**, and **BMP-18**, there is optimism that these derivatives may exhibit a stronger binding force and enhanced staining effects in bacterial applications.

2.2. Fluorogenic imaging of Gram-positive bacteria

The cell wall of bacteria primarily consists of a phospholipid bilayer and peptidoglycan, with variations in composition among different bacterial cell walls. In general, Gram-positive bacteria feature a single-cell membrane surrounded by a thick layer of peptidoglycan, while Gram-negative bacteria have a thin layer of peptidoglycan sandwiched between two layers of phospholipids, with an outermost lipopolysaccharide layer. In our study, *Bacillus cereus* (*B. cereus*) and *Escherichia coli* (*E. coli*) were chosen as representatives of Gram-positive and Gram-negative bacteria, respectively. The objective was to assess whether bacterial membranes could drive the disassembly of **BMP-alkyl** for bacterial fluorescence imaging. We added **BMP-alkyl** (2 μM) and the commercial bacterial membrane dye FM4–64 (2 μM) to a mixed bacterial solution of *E. coli* and *B. cereus* for 10 minutes, directly using it for

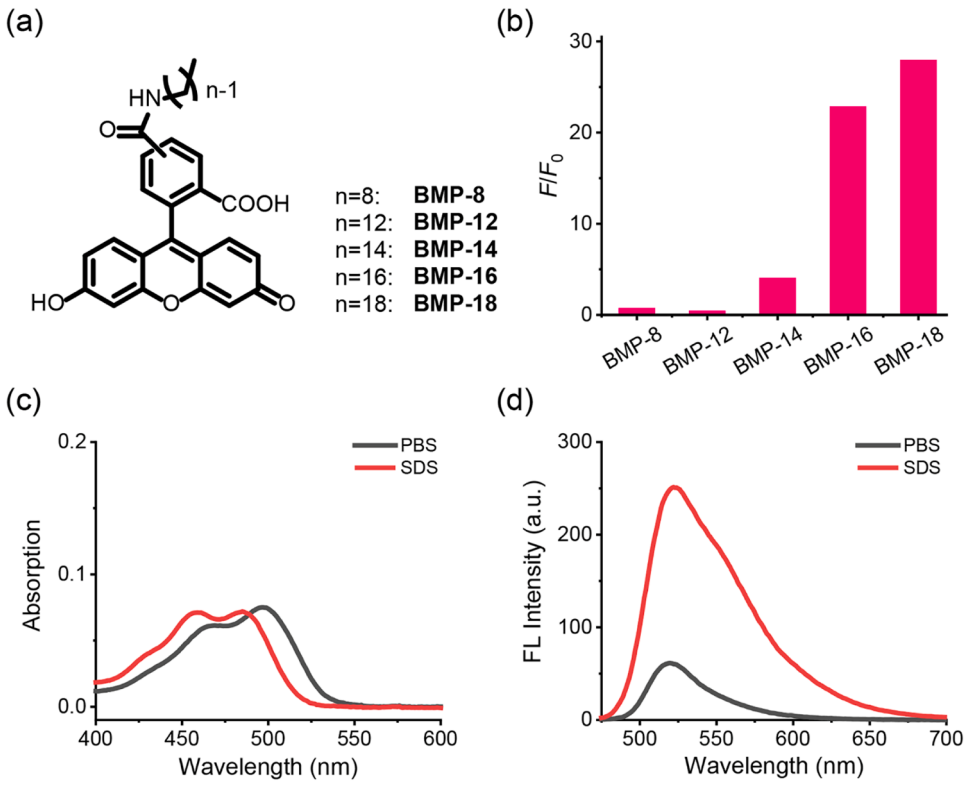


Fig. 1. (a) Chemical structure of **BMP-alkyl**. (b) Fluorescence intensity enhancement of different **BMP-alkyl** (5 μM) in response to 5 mM SDS in PBS (20 mM, pH = 7.4). F_0 was fluorescence intensity of **BMP-alkyl** in PBS (20 mM, pH = 7.4). (c) Absorption spectra of 5 μM **BMP-14** in the absence and presence of 5 mM SDS in PBS (20 mM, pH = 7.4). (d) Fluorescence spectra of 5 μM **BMP-14** in the absence and presence of 5 mM SDS in PBS (20 mM, pH = 7.4).

confocal imaging without washing.

Fig. 2a illustrates that, with the exception of **BMP-8**, the other four probes selectively labeled *B. cereus*, while *E. coli* exhibited minimal fluorescent signals. FM4–64 dyes, on the other hand, indiscriminately labeled bacterial membranes. The merged imaging allowed for easy differentiation between Gram-positive and Gram-negative bacteria under a fluorescence microscope, facilitating rapid bacterial type screening. Notably, in the presence of the membrane-competing dye FM4–64, the fluorescence signal of **BMP-12** was very weak. However, when bacteria were exclusively incubated with **BMP-12**, a strong fluorescence signal was detected specifically in Gram-positive bacteria *B. cereus*. This observation indicates that **BMP-12** possesses a weak binding ability to the cell membrane of Gram-positive bacteria.

To further validate the imaging selectivity of the **BMP-alkyl** series of probes against Gram-positive bacteria, we examined their staining capability against two other Gram-positive bacteria, *Staphylococcus aureus* (*S. aureus*) and *Listeria monocytogenes* (*LM*), as well as Gram-negative bacteria *Citrobacter freundii* (*C. freundii*) and *Acinetobacter baumannii* (*A. baumannii*), and the fungus *Candida tropicalis* (*C. tropical*). As depicted in Fig. 2b and Figure S6-S10, we observed bright fluorescent signals exclusively for Gram-positive bacteria (*S. aureus* and *LM*) when incubated with three probes (**BMP-14**, **BMP-16**, and **BMP-18**). Conversely, for Gram-negative bacteria *A. baumannii* and *C. freundii*, as well as the fungus *C. tropicalis*, no fluorescent signals were observed. The inability of **BMP-12** to image *LM* may be attributed to its poor binding capacity, explaining the lack of a fluorescent signal. SIM imaging of *B. cereus* membranes was successfully performed using **BMP-12**, **BMP-14**, and **BMP-16**, achieving resolution beyond the diffraction limit. This highlights the potential of these probes for dual-functional applications in both sterilization processes and real-time visualization of these sterilization events (Figure S11).

2.3. Antibacterial activity

We observed the interaction process of **BMP-alkyl** dyes with *E. coli* and *B. cereus* using confocal microscopy in the presence of propidium iodide (PI) to investigate the killing effect of different **BMP-alkyl** dyes on bacteria. PI is a commonly used fluorescent turn-on probe (emitting red fluorescence) for identifying dead bacteria, as it binds to nucleic acids after entering bacteria with compromised cell membranes. Hochesst33342, a commercial nucleic acid dye, was used to indicate bacteria non-discriminatively.

In Fig. 3, fluorescence images of **BMP-8** and bacteria mixed solutions (*E. coli* and *B. cereus*) in the presence of PI reveal almost no red fluorescent signal, suggesting that **BMP-8** had little effect on the membrane permeability of *E. coli* and *B. cereus*. This observation aligns with the idea that most anionic fluorescein molecules cannot approach the negatively charged membrane of bacteria due to electrostatic repulsion. Consequently, no green fluorescence is activated, resulting in a poor bactericidal effect. Under the same experimental conditions, the other four **BMP-alkyl** dyes, known to intercalate into bacterial membranes, were incubated with bacteria mixed solutions (*E. coli* and *B. cereus*) for 30 minutes in the presence of PI (Fig. 3). Green and red fluorescence signals were observed in all Gram-positive bacteria, indicating that the binding of the amphiphilic fluorescein derivatives to the *B. cereus* membrane led to the disruption of the bacterial cell wall integrity. However, no signals were detected in *E. coli*, implying that **BMP-alkyl** had no damaging effect on the bacterial membrane of *E. coli*. Simultaneously, we imaged mixed bacterial solutions using only PI dye, confirming that PI dye had no effect on bacteria under the specified conditions (Figure S12).

To validate the bactericidal effect of **BMP-alkyl**, we quantified the impact on bacteria by monitoring the kinetics of changes in PI fluorescence spectra. We utilized live and dead bacteria to observe variations in the fluorescence spectra of PI dyes. For live *E. coli* and *B. cereus*, the

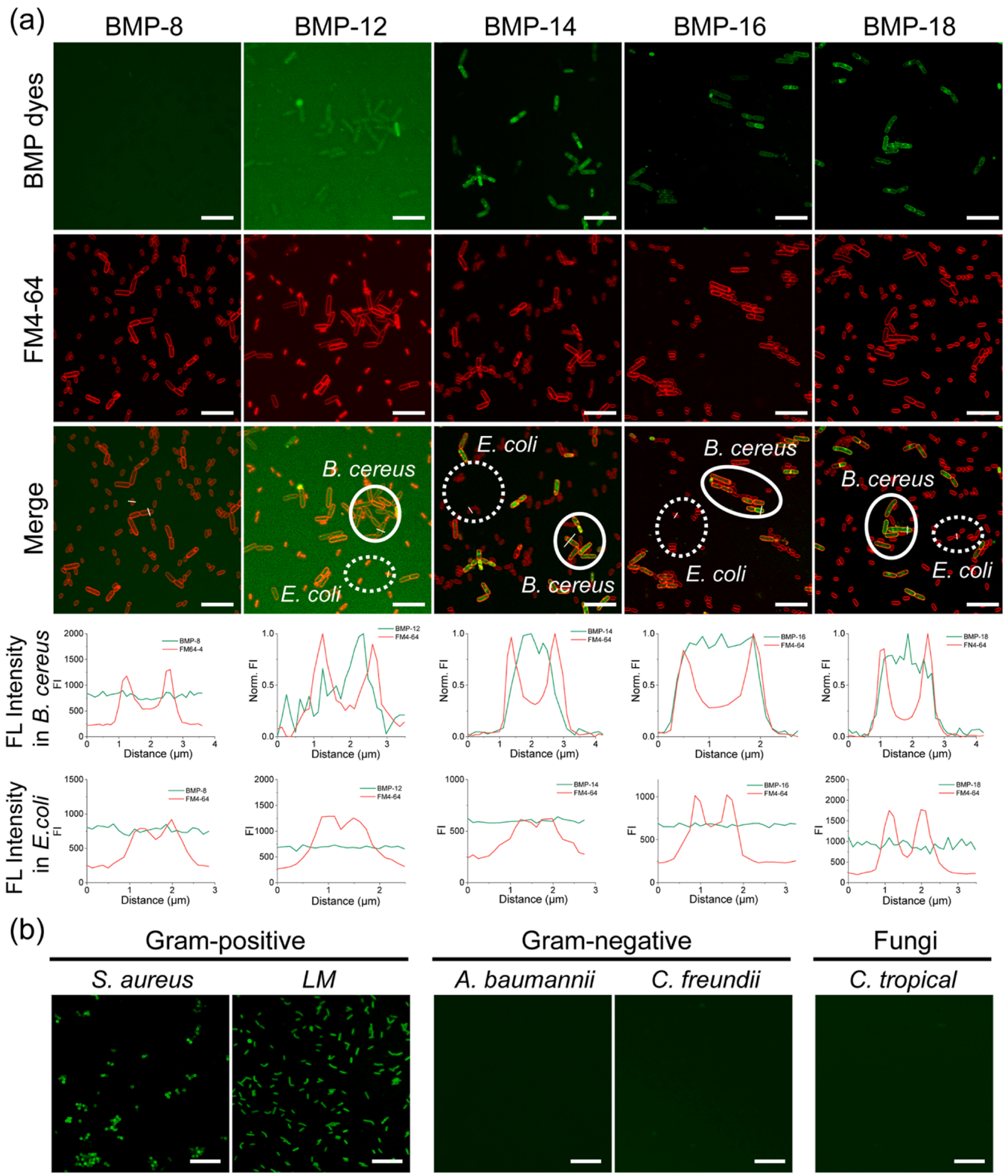


Fig. 2. (a) Confocal fluorescence wash-free imaging of Gram-positive bacteria in mixed solutions of *E. coli* and *B. cereus* by using different side chains' fluorescein **BMP-alkyl**. Bacteria mixed solutions stained with 2 μM **BMP-alkyl** compounds and 2 μM FM4-64 for 10 min in 37 °C. Upper: **BMP-alkyl** signal (Ex = 488 nm, pseudo green color); middle: FM4-64 signal (Ex = 543 nm, pseudo red color); bottom: overlay of upper and middle images; scale bar: 5 μm. And fluorescence intensity graphs normalized fluorescence profiles along the lines shown in merge images. (b) Universal adaptation for selective imaging of Gram-positive bacteria. Confocal fluorescence images of Gram-positive bacteria (*S. aureus* and *LM*; left two pictures), Gram-negative bacteria (*A. baumannii* and *C. freundii*; right: middle two pictures) and Fungi (*C. tropical*; right picture) after treatment with **BMP-14** (2 μM).

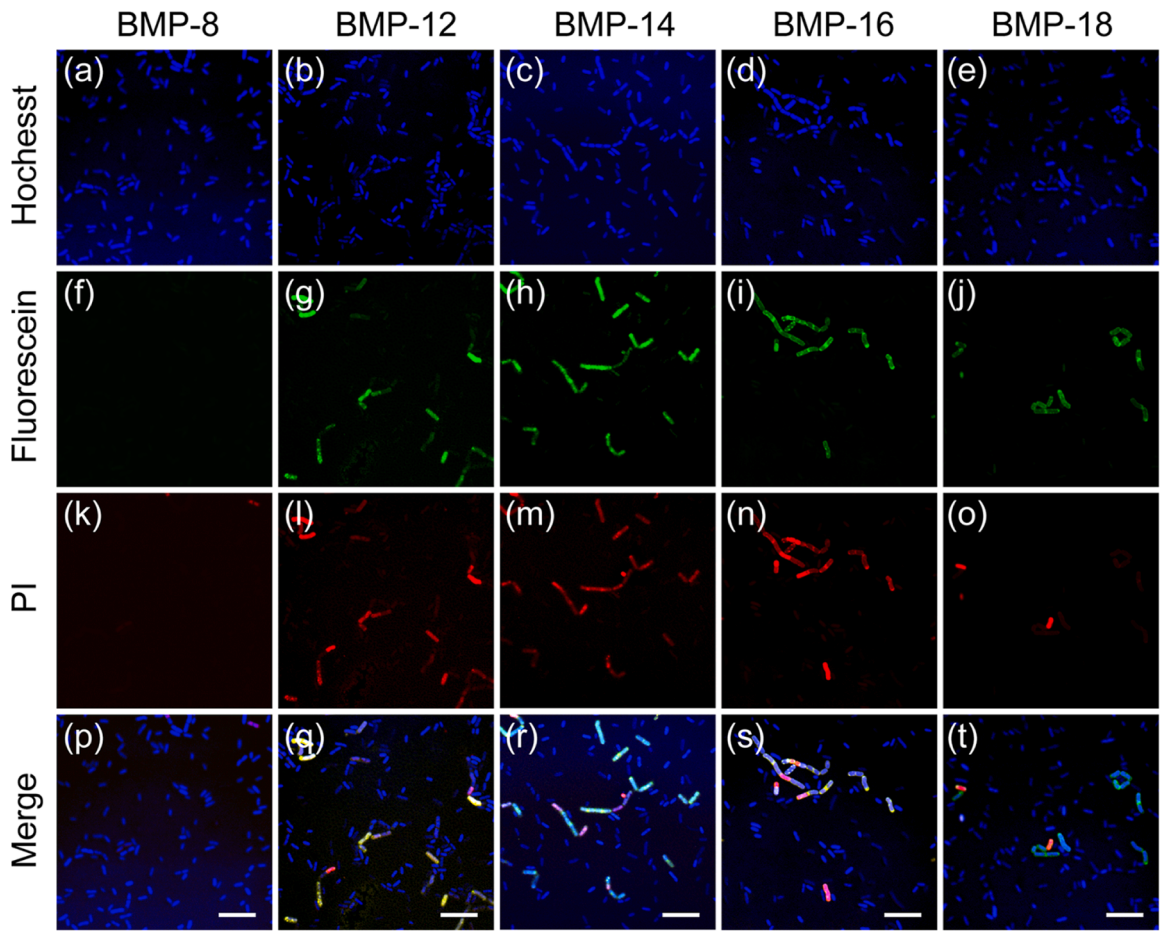


Fig. 3. BMP-alkyl probe for selective killing of Gram-positive bacteria. Confocal fluorescence images of mixed bacteria solutions (*E. coli* and *B. cereus*) after treatment with **BMP-alkyl** probe (10 μM), Hochesst33342 (2 μM) and PI (2 μM) for 30 min in 37 °C. **BMP-8** ((a),(f),(k),(p)), **BMP-12** ((b),(g),(l),(q)), **BMP-14** ((c),(h),(m),(r)), **BMP-16** ((d),(i),(n),(s)) and **BMP-18** ((e),(j),(o),(t)). Hochesst33342 Ex = 405 nm, **BMP-alkyl** (Fluorescein) Ex = 488 nm, PI Ex = 543 nm, scale bar: 5 μm.

fluorescence spectra resembled that of PBS buffer. The fluorescence intensity of dead *E. coli* and *B. cereus* was 6.5-fold and 5.1-fold higher than that of live bacteria, respectively (Fig. 4).

Subsequently, we examined the fluorescence spectra trend of PI dyes when **BMP-alkyl** was introduced to live bacteria. As illustrated in Fig. 4 and Figure S13-S16, **BMP-12** and **BMP-14** demonstrated a rapid bactericidal effect on *B. cereus*, essentially completing within 30 minutes. **BMP-16** and **BMP-18** exhibited a certain killing effect on *B. cereus*, albeit at a slower pace. Notably, none of the **BMP-alkyl** probes exhibited a bactericidal effect on *E. coli*. These findings indicate that **BMP-14** can accurately screen and identify Gram-positive bacteria while effectively eliminating them. To further assess the bactericidal efficacy of these probes against Gram-positive bacteria, we conducted minimum inhibitory concentration (MIC) tests (Table S2). Our findings revealed that **BMP-12** and **BMP −14** exhibited a pronounced inhibitory effect against Gram-positive bacteria, with a minimum inhibitory concentration as low as 16 μM. Additionally, **BMP-14** exhibits low toxicity to cells, demonstrating almost no adverse effects at bactericidal concentrations (Figure S17).

The remarkable antibacterial properties of **BMP-14** and **BMP-12** motivated us to delve deeper into the specific mechanism underlying their antibacterial process. As effective protonophoric uncoupler agents, fluorescein derivatives need to initially bind to the bacterial membrane and alter the potential of the bacterial membrane. Simultaneously, the anionic fluorescein molecule can induce a shift in the steady state of the bacterial membrane, leading to the destruction of the bacterial membrane and eventual cell death. To test this hypothesis, we first observed fluorescence images of Gram-positive bacteria *B. cereus* treated with **BMP-14** at different time points. Initially, **BMP-14** primarily concentrated on the bacterial membrane. After 30 minutes, **BMP-14** had permeated the entire bacteria (Fig. 5).

Subsequently, we measured changes in bacterial membrane potential using a commercial membrane potential dye, DiSC3(5). This membrane potential-sensitive dye spontaneously aggregates into the phospholipid bilayer, causing quenched fluorescence. When the membrane is depolarized and the membrane potential is lost, DiSC3(5) is released into the solution, resulting in fluorescence enhancement proportional to the decrease in membrane potential. From Fig. 5, it is evident that **BMP** can rapidly alter the bacterial membrane potential, with **BMP-12** and **BMP-14** exhibiting more pronounced effects than the other probes, aligning with their superior sterilization efficacy. The experimental results affirm our hypothesis that **BMP-alkyl** probes initially aggregate on the Gram-positive bacterial membrane, inducing changes in bacterial membrane potential, leading to the destruction of the bacterial membrane structure, and ultimately causing bacterial death.

3. Conclusions

In conclusion, we successfully designed and synthesized five amphiphilic fluorescein derivatives modified with different alkyl chains. The variations in alkyl chains result in different degrees of aggregation in aqueous solutions and binding capacities to negatively charged bacterial membranes, allowing for selective imaging and rapid sterilization of Gram-positive bacteria. Among these derivatives, **BMP-14** stands out

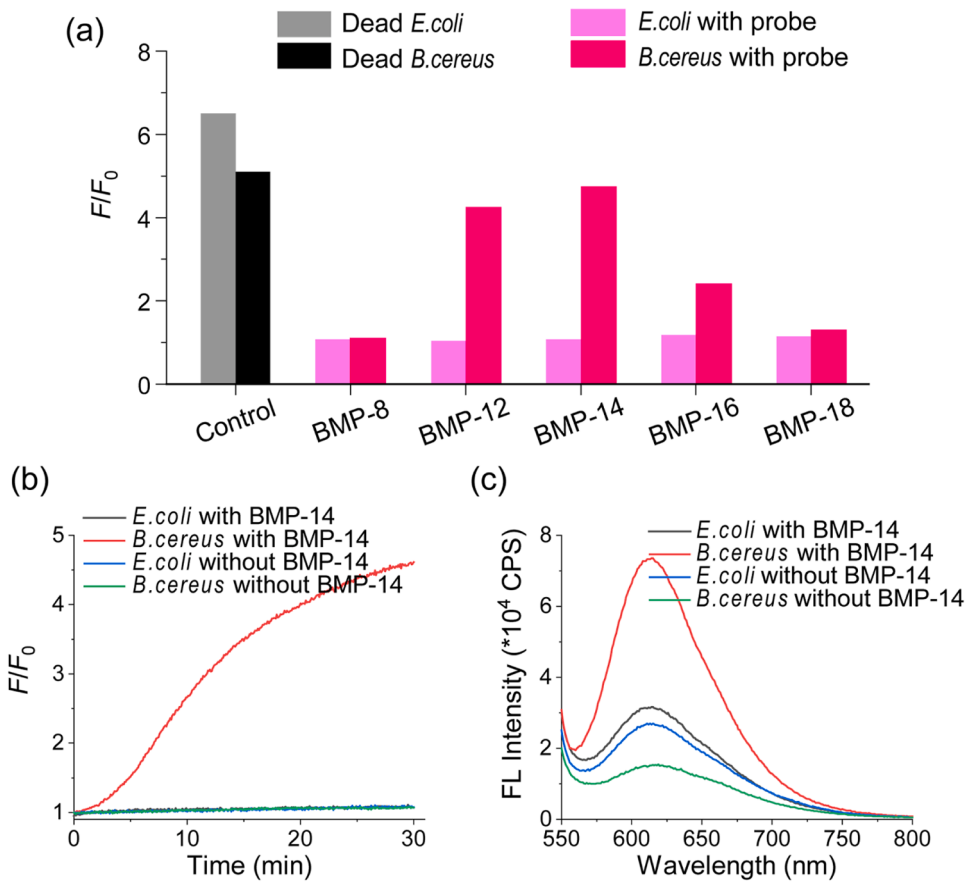


Fig. 4. (a) Normalized fluorescence intensity of 5 μM PI for living bacteria in PBS (20 mM, pH = 7.4). Grey is dead *E. coli* which pretreated with glycerol, black is dead *B. cereus* which pretreated with glycerol, magenta is living *E. coli* treated with **BMP-alkyl** (30 μM) for 30 mins, pink is living *B. cereus* treated with **BMP-alkyl** (30 μM) for 30 mins. (b) Time course of normalized fluorescence intensity of PI in the absence and presence of 30 μM **BMP-14** for living bacteria solutions (*E. coli* or *B. cereus* in PBS). (c) Fluorescence spectra of 5 μM PI in the absence and presence of 30 μM **BMP-14** in PBS (20 mM, pH = 7.4) added living bacteria. OD_{600} = 0.5.

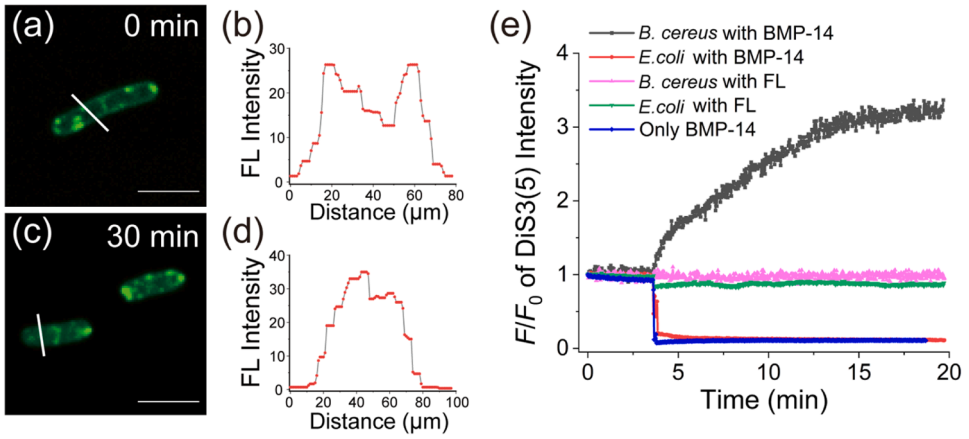


Fig. 5. Sterilization mechanism of **BMP-alkyl** probes. Confocal fluorescence images of *B. cereus* treated with 30 μM **BMP-14** at different time, (a) was 0 min, (c) was 30 min. Scale bar: 5 μm. (b) and (d) are the fluorescence intensity profile of a and c regions, respectively. (e) Time course of normalized fluorescence intensity of DiSC3(5) in the absence and presence of 30 μM **BMP-14** for living bacteria solutions (*E. coli* or *B. cereus* in PBS), OD_{600} = 0.5.

as a universal agent for selectively imaging Gram-positive bacteria, demonstrating the additional capability of quick bacterial eradication within 30 minutes. The sterilization mechanism of **BMP-14** involves its rapid binding to the cell membrane of Gram-positive bacteria, continuous accumulation, and subsequent transport into the bacterial membrane. This process leads to the elimination of the membrane potential and the destruction of the membrane structure, culminating in the rapid demise of bacteria. In essence, the membrane-driven disassembly of these amphiphilic fluorescein derivatives adeptly integrates the targeting, imaging, and killing functions for Gram-positive bacteria. This makes them highly attractive for applications in bacterial diagnosis and therapy.

CRediT authorship contribution statement

wenjuan Liu: Data curation. **Lu Miao:** Writing – review & editing, Funding acquisition. **Zhaochao Xu:** Writing – review & editing, Writing – original draft, Funding acquisition, Conceptualization. **Wei Zhou:** Writing – original draft, Investigation, Data curation. **Ning Xu:** Data curation. **Jie Chen:** Data curation. **Xiangning Fang:** Data curation. **Qinglong Qiao:** Writing – original draft, Supervision, Funding acquisition. **Yi Tao:** Data curation. **Chengen Duan:** Data curation. **Jin Li:** Data curation.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.snb.2024.135691.

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