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FULL PAPER

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Wash-free, peptide-based fluorogenic probes for microbial imaging

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Abstract

Peptides with pan-antimicrobial affinity were synthesized and decorated with environmentally sensitive fluorophores nitrobenzoxadiazole (green) and merocyanine (red). The labeling efficacies against a range of clinically relevant fungal and Gram-negative and positive bacterial species (*Candida albicans*, *Escherichia coli*, and *Staphylococcus aureus*) were explored. The fluorogenic probes containing exclusively L or D-amino acids showed rapid and efficient labeling of all microbial species, whereas probes with a mixture of L or D-amino acids failed to label fungi or bacteria, highlighting the importance of the α-helical peptide structure for interaction with the microbial cell membrane. Importantly, the nature of the dye allowed fluorescence detection/labeling without the need for a wash step, paving the way for direct application of the probes.

KEYWORDS

antimicrobial peptide, bacteria, fluorescent probe, fungi, optical imaging

1 | INTRODUCTION

Late-stage diagnosis or misdiagnosis and assumption of microorganism-based diseases results in enormous health problems, economic burden and social complications, while leading to overuse and misuse of antibiotics that drive antimicrobial resistance.[1] Current diagnostic approaches typically require sampling and culturing, which impose delays in the identification of the specific antimicrobial infection.[2] The polymerase chain reaction is a powerful diagnostic tool, due to its high sensitivity and selectivity, but requires predictive knowledge of the pathogen it will detect and identify, while the sophisticated instrumentation and requirements for cold-chains means it will be inaccessible in developing countries where the bulk of microorganism-based infections reside.[3,4] Thus, new, simple, and affordable diagnostic methods are needed to allow rapid and specific diagnosis of bacterial and fungal infections.

Optical molecular imaging has attracted much attention as a method for early stage diagnosis due to its versatility and power for detecting disease-related biomarkers and pathogens both *in vitro* and in real-time *in vivo*. Importantly, optical molecular imaging can provide information at a molecular level at low cost and simplicity.[5–7] In targeted optical molecular imaging, rationally designed probes, comprised of a fluorescent reporter conjugated to target-specific moiety (e.g., an antibiotic), provide high sensitivity and selectivity. The applied fluorophores typically fall into two categories: so-called "always-on" or those that are "turned-on" upon probe-target interaction. The "turn-on" probes often utilize fluorogenic dyes that are weakly fluorescent until there is a small change in their local environment

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(eg, polarity, hydrophobicity, or viscosity).[8] Examples of these dyes include nitrobenzoxadiazole (NBD) and merocyanine (MeroCy), which show weak fluorescence in an aqueous environment and produce a much stronger signal, for example, upon binding to a hydrophobic target. This reduces background fluorescence and eliminates the need for wash steps, which is particularly important in diagnostic applications. This environment-based activation strategy makes them outstanding candidates for probes for optical imaging of microorganisms as cell membranes are hydrophobic.

The interest in antimicrobial peptides (AMPs) has increased significantly[9–11] with the current AMP database containing over 3150 entries from prokaryotic and eukaryotic organisms, including 1135 naturally isolated antifungal peptides.[12] Most AMPs act by disrupting the cell membrane by various mechanisms, with important structural features including the length of the peptide, overall charge, hydrophobic/hydrophilic properties, and even the length of the side chain.[13] For example, Ng *et al*. screened 61 short (3-11 residues) AMPs for antifungal activity against fluconazole-resistant strains of *Candida albicans* and established structure-activity relationships, with the amphipathic peptide H-KKLIKKILKIL-NH_2 (P11-6)[14] showing better antifungal activity than the antifungal drug miconazole.[15] Peptides, including AMPs, have also gained interest as targeting moieties for imaging applications.[16–18]

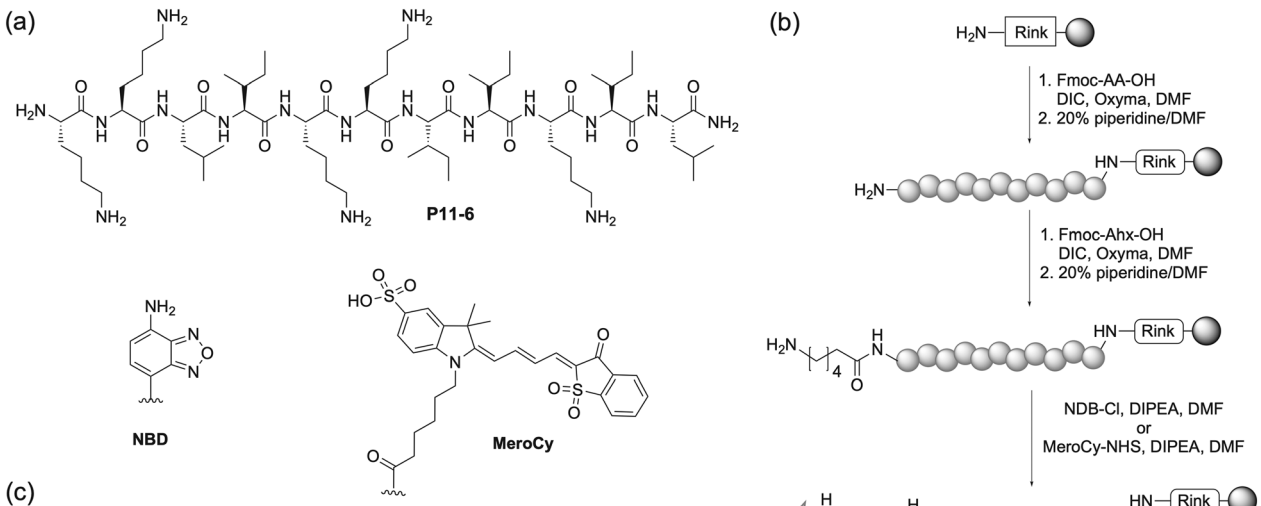
Here, we report a series of fluorescent probes based on the AMP P11-6, with the probes carrying an environmentally sensitive fluorophore NBD (green, $\lambda_{ex/em}$ 485/545 nm) or MeroCy (red, $\lambda_{ex/em}$ 595/630 nm) that only "turn-on" on/in hydrophobic microbial membranes (Figure 1). These probes were applied in the imaging of clinically relevant microorganisms including *C. albicans* (fungus), *Escherichia coli* (Gram-negative bacteria), and *Staphylococcus aureus* (Gram-positive bacteria), and their mechanism of action explored by investigating the effect of L and D amino acid residues on selectivity, with the synthesis of six analogues of P11-6.

2 | METHODS

2.1 | General information

Chemicals were purchased from Sigma Aldrich, Merck, Acros, VWR, GL Biochem, and Fisher Scientific and used without further purification. NMR spectra were recorded at 298 K in deuterated solvents using a Bruker AVA500 spectrometer operating at 500 MHz for ^{1}H and 126 MHz for ^{13}C, with chemical shifts reported in ppm (referenced to residual non-deuterated solvent for ^{1}H and deuterated solvent for ^{13}C). Fluorescence spectra were measured on a Shimadzu RF-6000 spectrofluorophotometer using a quartz cuvette (10 mm). High-resolution mass spectra (HRMS) were recorded on a Bruker 3.0 T Apex II spectrometer 1.

Analytical reverse-phase high-performance liquid chromatography (RP-HPLC) was performed on an Agilent 1100 system equipped with a Phenomenex Kinetex 5 μm XB-C18 100 Å LC Column



(c)

Probe	Fluorophore	Spacer	Sequence
NBD-L	NBD	6-Ahx	KKLIKKILKIL-NH_2
NBD-D	NBD	6-Ahx	kklikkilkil-NH_2
NBD-LD	NBD	6-Ahx	KKliKKilKil-NH_2
NBD-DL	NBD	6-Ahx	kkLIkkILkIL-NH_2
MeroCy-L	MeroCy	6-Ahx	KKLIKKILKIL-NH_2
MeroCy-D	MeroCy	6-Ahx	kklikkilkil-NH_2

FIGURE 1 Design and synthesis of the fluorogenic microbial probes based on the AMP P11-6. A, Structures of P11-6, and environmental fluorophores nitrobenzoxadiazole (NBD) and merocyanine (MeroCy) used in this study. For the synthesis of MeroCy-CO_2H, see supporting information (Scheme S2). B, Solid-phase synthesis of the fluorescently labeled peptides. C, The library of imaging probes based on P11-6

(50 × 4.6 mm) with a flow rate of 1 mL/min. Compounds were eluted with a gradient of 5% of CH_3CN in H_2O to 95% of CH_3CN (with 0.1% HCO_2H) over 6 minutes, followed by 4 minutes isocratic elution, with detection at 495 or 600 nm. Semipreparative RP-HPLC was performed on an Agilent 1100 system equipped with a Zorbax Eclipse XDB-C18 RP column (250 × 9.4 mm, 5 μm) with a flow rate 2.0 mL/min, eluting with a gradient of 5% of CH_3CN in H_2O to 95% of CH_3CN (with 0.1% HCO_2H) over 25 minutes, followed by isocratic period of 5 minutes.

2.2 | Synthesis of MeroCy-CO_2H

(2*E*)-2-[(2*E*)-3-methoxyprop-2-enylidene]-1-benzothiophen-3(2*H*)-one 1,1-dioxide[19,20] (222 mg, 0.89 mmol) and 1-(5-carboxypentyl)-2,3,3-trimethyl-3*H*-indolium-5-sulfonate[21] (315 mg, 0.89 mmol) were mixed in a microwave vial with NaOAc (73 mg, 0.089 mmol) in MeOH/DCM (4 mL, (1:1, vol/vol). The vial was sealed and heated to 75°C for 30 minutes under microwave irradiation (Biotage Initiator 2.0.). The reaction mixture was cooled to room temperature and the solvent evaporated under reduced pressure. The crude compound was purified by preparative HPLC to give MeroCy-CO_2H as a purple solid. Yield 26% (132 mg). Purity (HPLC, 600 nm detection): >97%. t_R 3.95 minutes. ^1H NMR (500 MHz, CD_3OD, δ, ppm) δ 8.12 (t, *J* = 12.9 Hz, 1H), 7.97 (m, 1H), 7.93 (m, 1H), 7.91 to 7.80 (m, 5H), 7.21 (d, *J* = 8.5 Hz, 1H), 6.82 (t, *J* = 13.2 Hz, 1H), 6.23 (d, *J* = 13.3 Hz, 1H), 4.04 (t, *J* = 7.5 Hz, 2H), 2.32 (t, *J* = 7.2 Hz, 2H), 1.81 (p, *J* = 7.5 Hz, 2H), 1.72 (s, 6H), 1.70 to 1.61 (m, 2H), 1.49 (p, *J* = 7.7, 7.1 Hz, 2H); ^{13}C NMR (125 MHz, CD_3OD, δ, ppm) δ 177.5, 172.4, 145.5, 144.8, 142.2, 141.9, 136.0, 134.9, 127.9, 124.5, 121.4, 121.2, 120.1, 110.6, 102.6, 49.8, 44.6, 34.6, 28.2, 27.9, 27.4, 25.8; LC-MS (ESI, *m/z*) $[M + H]^+$ 572.0, $[M + Na]^+$ 594.1; HR-MS (ESI, *m/z*): calc. $C_{28}H_{30}NO_8S_2$ 572.14074; found: 572.14270 $[M + H]^+$.

2.3 | Solid-phase synthesis

All the peptides were synthesized on an aminomethyl polystyrene resin (0.745 mmol/g, 1% DVB, 100-200 mesh) functionalized with an Fmoc-Rink linker. The peptides were synthesized using 500 mg of resin, and for each reaction, the resin was preswollen in DCM. The coupling reactions were monitored by the Kaiser test.

2.3.1 | Fmoc-Rink linker/amino acid coupling

A solution of the Fmoc-Rink linker or the appropriate *N*-Fmoc protected amino acid (3.0 equiv. per amine) and Oxyma (3.0 equiv.) in DMF (0.1 M) were stirred for 10 minutes, after which DIC (3.0 equiv.) was added and the mixture stirred for 1 minute. The preactivated mixture was added to the resin and the reaction was shaken for 3 hours. The solution was drained, and the resin washed thrice with DMF, DCM, and MeOH.

2.3.2 | Fmoc cleavage

The resin was shaken with 20% piperidine in DMF (2 × 10 minutes). The solution was drained and the resin washed thrice with DMF, DCM, and MeOH.

2.3.3 | MeroCy-CO_2H coupling

A solution of MeroCy-CO_2H (1.5 equiv. per amine) and HSPyU (1.5 equiv.) in DMF (0.1 M) was stirred for 2 hours at 40 °C (the formation of the NHS ester was monitored by analytical HPLC). After completion, the mixture was added to the resin and shaken overnight. The solution was drained and the resin was washed thrice with DMF, DCM, and MeOH.

2.3.4 | NBD-Cl coupling

A solution of NBD-Cl (3.0 equiv. per amine) and DIPEA (3.0 equiv.) in DMF (0.1 M) was added to the resin and the reaction mixture shaken overnight. The solution was drained and the resin washed thrice with DMF, DCM, and MeOH.

2.3.5 | Deprotection and peptide cleavage

The resin was shaken in TFA/TIS/H_2O (95:2.5:2.5) for 3 hours at a ratio of 100 mg resin to 1 mL of the cleavage cocktail. The filtrate was collected, and the product was precipitated into ice-cold diethyl ether, collected by centrifugation (repeated twice). The crude peptides were dissolved in H_2O/CH_3CN (1:1, vol/vol) and purified by semi-preparative HPLC, and analyzed by HPLC and HRMS (see Supporting Information).

2.4 | Fluorescence measurements

Stock solutions of the probes (1.0 mM in PBS) were diluted to a final concentration of 1.0 μM for MeroCy-L and 5.0 μM for NBD-L using either DMSO or PBS. The fluorescence emissions of the solutions were measured using a RF-6000 spectrofluorophotometer (Shimadzu) upon excitation at 475 nm (NBD-L) and 560 nm (MeroCy-L). For the fluorescent excitation spectra, emissions at 550 nm (NBD-L) and 630 nm (MeroCy-L) were measured.

2.5 | Fungal and bacterial cell culture

C. albicans (ATCC MYA-2876) were grown from −80 °C frozen bead overnight at 37 °C in a shaking incubator in liquid Potato dextrose media broth (10 mL). *E. coli* (ATCC 25922) and *S. aureus* (ATCC 25923) single colonies were taken from agar plates and incubated

overnight at 37 °C in LB liquid media (10 mL) in a shaking incubator. The overnight cultures were centrifuged at 3000 rpm for 10 minutes and the pellet resuspended in 1 mL of PBS. The cells were placed into an Eppendorf vial (1.5 mL) and centrifuged at 13 500 rpm for 2 minutes. The supernatant was removed and the cells were resuspended in fresh PBS (1 mL) and diluted further with PBS to obtain an OD_{600} of 0.4.

2.6 | Fungal and bacterial microscopy

μ-Slide eight-well confocal chambers (ibidi) were coated with poly-D-lysine (0.1 mg/mL in PBS) for 20 minutes at 37 °C, after which the wells were washed with PBS. Microbial "cells" were placed into the wells (at OD_{600} of 0.2) and incubated with the probes (10 μM in PBS, n = 2 individual experiments) in the dark at 37 °C for 20 minutes. Imaging was performed on a Leica TCS SP8 laser scanning confocal microscope (λ_{ex} = 488 nm for NBD-based probes, λ_{ex} = 561 nm for merocyanine-based probes). Images were analyzed using ImageJ2 (National Institutes of Health).

2.7 | Circular dichroism spectroscopy

The circular dichroism (CD) spectra of the peptides (dissolved in 50% trifluoroethanol in 10 mM NaH_2PO_4 [pH 7.0]) were recorded between 185 and 260 nm at 50 nm/min; data pitch 0.1 nm; bandwidth 1 nm, response time 1 second in a 0.1 cm path length quartz cuvette at 25 °C (JASCO-810 spectropolarimeter). All spectra are an average of 25 measurements, acquired under the same conditions, and corrected by subtracting the buffer baseline.

3 | RESULTS AND DISCUSSION

3.1 | Design and synthesis of AMP-based fluorescent probes

The AMP H-KKLIKKILKIL-NH_2 (P11-6)[14] and, to improve proteolytic stability, its corresponding enantiomer (D-amino acid sequence) were synthesized on a Rink-amide functionalized polystyrene resin using an Fmoc/^{t}Bu-based solid-phase peptide synthesis, with DIC/Oxyma as the coupling combination (Figure 1B). NBD or merocyanine dye (Scheme S2) was attached to the amino-terminus of the peptide, after the addition of aminohexanoic acid (Ahx) as an aliphatic spacer. The probes (NBD-L, NBD-D, MeroCy-L, and

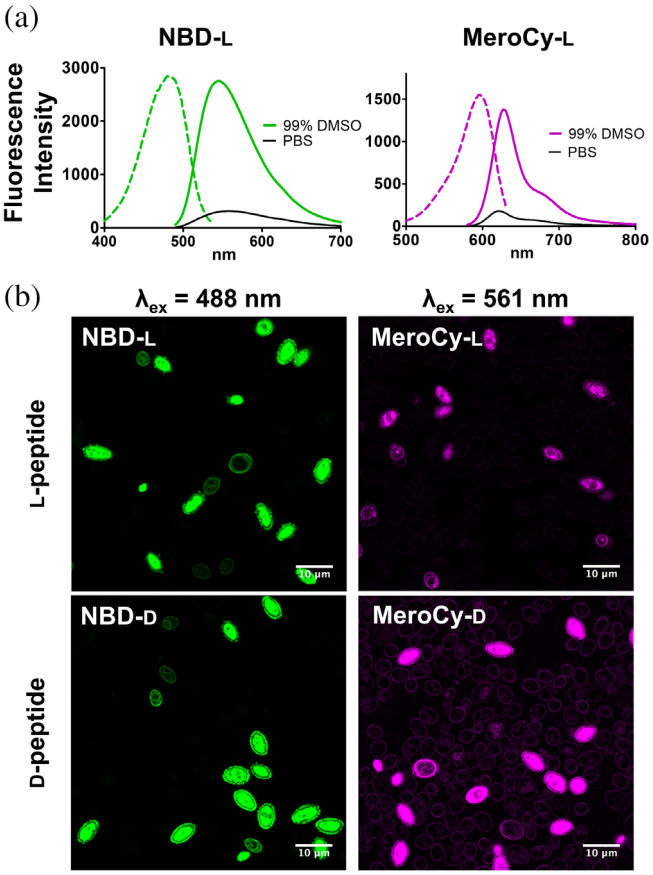


FIGURE 2 A, Excitation (dashed trace) and emission spectra of nitrobenzoxadiazole (NBD)-L (5 μM, $\lambda_{ex/em}$ = 480/545 nm) and MeroCy-L (1 μM, $\lambda_{ex/em}$ = 595/630 nm) in hydrophilic (PBS) vs a more hydrophobic environment (99% DMSO). Fluorogenic properties were evaluated by comparing the fluorescence intensities at the maximum emission wavelengths. B, Fluorescent confocal microscopy images of *C. albicans* labeled using the probes NBD-L and NBD-D (λ_{ex} = 488 nm) and MeroCy-L and MeroCy-D (λ_{ex} = 561 nm). Fungi were grown in potato dextrose liquid media overnight, then incubated with the probes (10 μM in PBS) for 20 minutes at 37 °C, and imaged. Scale bar 10 μm

TABLE 1 Characterization of the fluorogenic peptides. See Supporting Information for HPLC traces and mass spectra

	HPLC			HRMS		
Probe	t_R (min)	Detection (nm)	Purity	Formula	Calculated	Found
NBD-L	3.39	495	99%	$C_{78}H_{141}N_{21}O_{15}$ $[M + Na]^+$	1635.0808	1635.0796
NBD-D	3.42	495	99%	$C_{78}H_{141}N_{21}O_{15}$ $[M + Na]^+$	1635.0808	1635.0796
MeroCy-L	3.31	600	98%	$C_{100}H_{167}N_{19}O_{19}S_2$ $[M + Na]^+$	2025.2019	2025.2011
MeroCy-D	3.73	600	99%	$C_{100}H_{167}N_{19}O_{19}S_2$ $[M + Na]^+$	2025.2019	2025.2028
NBD-LD	2.50	495	97%	$C_{78}H_{141}N_{21}O_{15}$ $[M + Na]^+$	1635.0808	1635.0820
NBD-DL	2.64	495	97%	$C_{78}H_{141}N_{21}O_{15}$ $[M + H]^+$	1613.0989	1613.0981

Abbreviations: HPLC, high-performance liquid chromatography; HRMS, high-resolution mass spectra; NBD, nitrobenzoxadiazole.

MeroCy-D) were deprotected and cleaved off the linker (TFA/TIS/H_2O), purified by semipreparative HPLC, and analyzed by HPLC and HRMS (Table 1).

The environmentally sensitive nature of these probes was demonstrated by fluorescence analysis in PBS and in 99% DMSO, with a 9.2 (λ_{em} = 545 nm) and 8.5-fold (λ_{em} = 630 nm) increase in fluorescence intensities observed for NBD-L and MeroCy-L, respectively, in DMSO compared to PBS (Figure 2A).

3.2 | D and L-analogues of the probes label fungi, Gram-negative, and Gram-positive bacteria

The AMP P11-6 has been shown to have an MIC of 3.1 μM against a fluconazole-resistant strain of *C. albicans*.[15] Thus, the ability of NBD-L and NBD-D (probes with the fluorophore NBD attached to D and L variants of P11-6) to label *C. albicans* was first investigated with optimization of the probe concentration (Figure S1). Both the D and L-peptides rapidly labeled the membrane of the fungi, without a need for washing steps, in a concentration-dependent manner with fluorescence saturation observed at <10 μM. Although the NBD fluorophore (λ_{ex} = 485 nm) offers advantages (e.g., environmental sensitivity with a high signal to background ratio) over other always-on reporters (e.g., fluorescein), its emission window is in the green autofluorescence window of tissue. To overcome this, the red emissive (λ_{ex} = 595 nm) environmentally sensitive merocyanine dye[19,20,22] (MeroCy-CO_2H that bears acceptor and donor moieties connected to each other by double bonds) was also conjugated onto P11-6. Similar labeling efficacies were observed for the merocyanine-conjugated probes with no differences observed between the D and L peptides (Figure 2B). Next, bacterial labeling was investigated using Gram-positive Methicillin sensitive *S. aureus* and Gram-negative *E. coli*. The probes NBD-L, NBD-D, MeroCy-L, and MeroCy-D all efficiently labeled bacteria proving that these probes can be utilized for the broad-spectrum labeling of different microbial species (Figure 3). At 10 μM, all the species were efficiently labeled, whereas at lower concentrations Gram-positive *S. aureus* showed better binding of the probes (Figure S4). The probes did not bind to mammalian epithelial cells (Figure S5).

3.3 | α-Helical conformation is required for the microbial labeling

The suggested mechanism of action of P11-6 is based on membrane disruption, explained by the Shai-Matsuzaki-Huang model,[23,24] with initial binding explained by the electrostatic interactions between the positively charged peptide and the negatively charged cell membrane phospholipids. Peptide P11-6 has been shown to exhibit amphipathic behavior and adopt an α-helical conformation,[14] with the α-helical conformation positioning the cationic residues along one face of the helix. Upon binding to the outer leaflet, the AMPs expand the membrane, allowing interaction with the inner layer of the cell membrane (forming pores), and eventually either disrupt the membrane or bind to intracellular targets. To obtain a more detailed understanding of the labeling mechanism and support this model, additional probes, NBD-LD (using L-lysine and all the other amino acids having the D-configuration) and NBD-DL (using D-lysine and all the other amino acids having the L-configuration), were synthesized and investigated for their labeling efficacies. The rationale was this would disrupt the formation of the α-helix and thus hinder the interaction between the probe and microorganisms' membrane. CD measurements showed that NBD-LD and NBD-DL lacked secondary structure

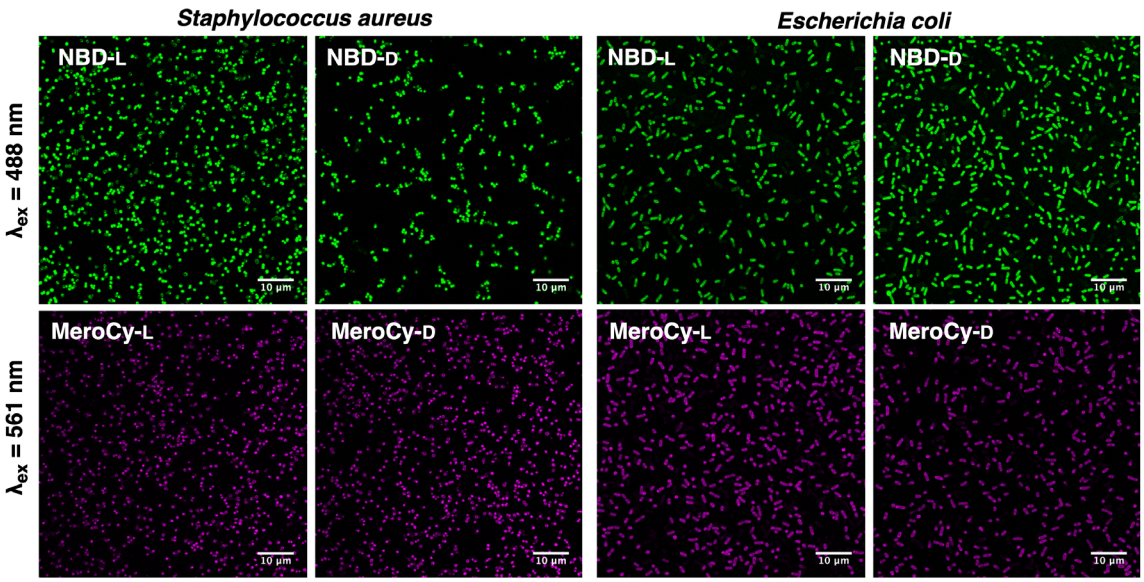


FIGURE 3 Fluorescent confocal microscopy images of *E. coli* and *S. aureus* labeled using the probes NBD-L and NBD-D (λ_{ex} = 488 nm) and MeroCy-L and MeroCy-D (λ_{ex} = 561 nm). Bacteria were grown in LB liquid media overnight, then incubated with the probes (10 μM in PBS) for 20 minutes at 37 °C, and imaged. Scale bar 10 μm

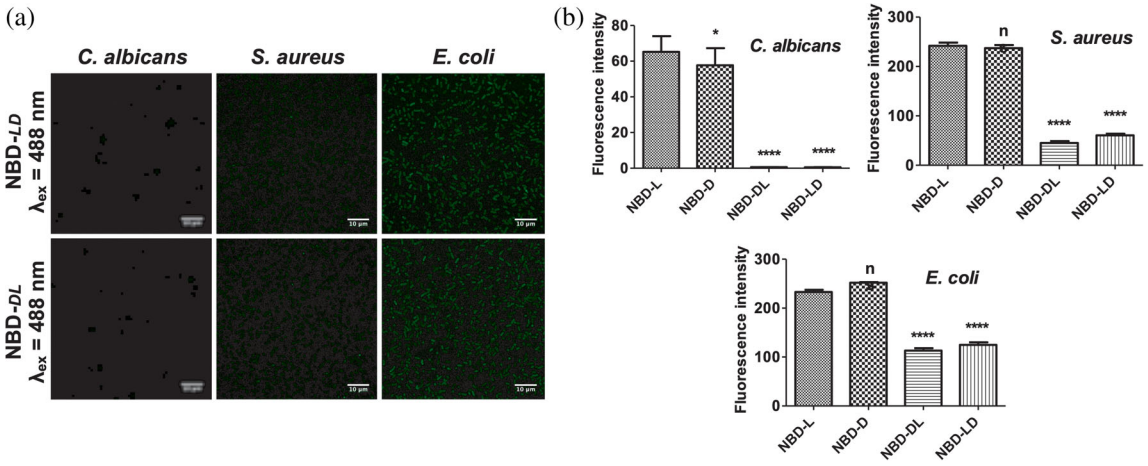


FIGURE 4 Labeling of fungi and bacteria with mixed configuration LD and DL probes. A, Fluorescent confocal microscopy images of fungi and bacteria labeled with NBD-LD and NBD-DL. Cells were incubated with the probes (10 μM in PBS) for 20 minutes at 37 °C and imaged (λ_{ex} = 488 nm). Scale bar 10 μm. B, Fluorescence intensities were quantified by single cell selection on a bright-field view and measuring the mean intensity of selection on raw fluorescent images (for at least 20 cells). Bars show the mean fluorescence (±SEM). *P < .05, ****P < .0001 by Bonferroni's multiple comparison test against NBD-L probe

compared to NBD-L and NBD-D (as evidenced by much reduced CD effect), while the peptides composed exclusively of L or D-amino acids showed α-helical characteristics as a convincing mirror pair (ESI, Figure S2).

When *C. albicans* was treated with NBD-LD and NBD-DL, under the optimized labeling conditions, no labeling of the fungi was observed (Figures 4 and S3), supporting the Shai-Matsuzaki-Huang model that a defined α-helical conformation is required for the membrane interactions and subsequent antifungal activity. Similarly, only low levels of membrane labeling were observed with *S. aureus* and *E. coli* compared with the single enantiomer peptides, although this was not as significant as lack of fungi labeling.

4 | CONCLUSIONS

A library of environmentally sensitive fluorogenic probes, based on an AMP, was synthesized and the probes investigated for their labeling efficacy toward bacteria and fungi. Fluorophore conjugated peptides synthesized exclusively with either L or D-amino acids showed excellent broad-spectrum labeling efficacies (fungi and bacteria), whereas the probes containing mixtures of L and D-amino acids showed no or reduced labeling of microorganisms, suggesting that the labeling mechanism follows the Shai-Matsuzaki-Huang model. This proof-of-concept study shows that peptides can be used as targeting moiety for a range of pathogens, with fluorogenic probes eliminating undesired background fluorescence. Fluorescent labeling of microbes has potential applications in cost-effective and rapid diagnosis (e.g., via imaging or cytometry) of microbial infections (e.g., in urine or sputum) or detection of microbes in other samples, with high sensitivity (compared, e.g., to bright-field imaging).

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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