A deep-red fluorogenic antimicrobial peptide for rapid wash-free staining and detection of bacteria

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Abstract: Fluorescent probes for rapid staining and detection of bacteria are needed for the diagnostics of bacterial infections as a measure for mitigating the dissemination of antibiotic resistance. Here we report the design, synthesis and characterization of bacteria-targeting fluorogenic peptides incorporating **Alared**, an environmentally sensitive fluorescent unnatural amino acid based on the Nile Red fluorophore. The new peptide-based probe named **UNR-1** displayed deep-red emission (λ_{max} = 665 nm) and enabled rapid add-and-read fluorescence staining of Gram-positive and Gram-negative bacteria under no-wash conditions, while also exhibiting selectivity over mammalian cells. The probe was compatible with fluorescence microscopy and flow cytometry and was used for the detection of *E. coli* in conditions mimicking bacteriuria.

Keywords: fluorescent probe, fluorogenic probe, bacterial detection, antimicrobial peptide, flow cytometry

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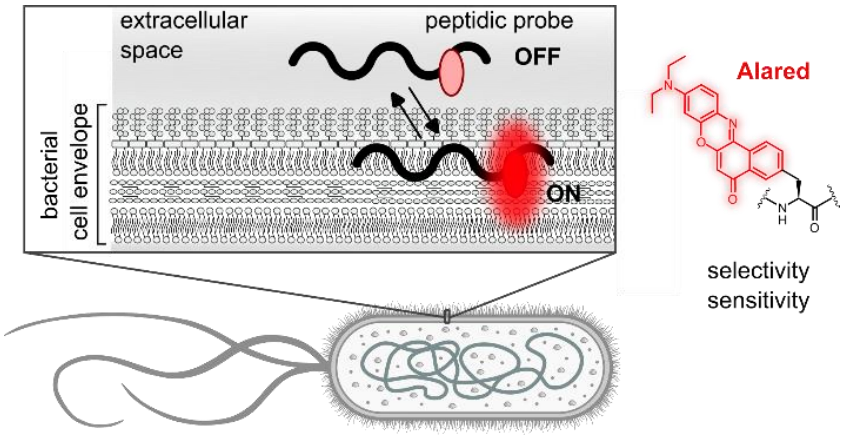

The worldwide spread of antibiotic-resistant bacteria poses a global threat to human health which must be addressed at multiple levels.[1–3] While the development of new antimicrobials is resource-intensive

and slow, new sensors and assays are needed for fast, sensitive and accurate detection of bacteria in biological samples as an essential strategy for preventing and managing bacterial infections. Such sensors and assays would be of great practical utility in clinical laboratory diagnostics, fundamental biomedical research on host-pathogen interactions, environmental and quality control testing, and other applications. Ultimately, such sensors could moderate the overuse of antibiotics and alleviate the spread of antibiotic resistance.[4–11] Biosensing platforms based on fluorescence readout offer distinct advantages owing to the possibility for rapid analysis, high sensitivity, facile multiplexing, and compatibility with live cells and tissues. In addition, fluorescence analysis can be performed using a wide range of instruments, including portable devices for point-of-care diagnostics. Given that, bacteria-specific fluorescent molecular probes have received substantial attention as prospective bioanalytical tools for fundamental research and for clinical diagnostics and imaging.[12–17]

A key advantage offered by the technology of fluorescent molecular probes is the possibility to combine of desired optical properties with high target selectivity, high signal-to-noise ratios and low limit of detection by tailoring the molecular architecture of the probe. In this context, fluorogenicity refers to the ability of a probe to exhibit enhanced fluorescence upon interaction with the target as compared with the free probe in solution.[18–20] Rationally designed fluorogenic molecular probes are well suited for robust detection of low-abundance targets with high signal-to-noise ratios in biological samples.[21–23] Ideally, the assay should be performed with minimal sample processing in an "add-and-read" format.

In the field of bacterial pathogen detection, fluorescent and especially fluorogenic probes hold great promise compared to conventional microbiological assays.[13–16,24] The conventional assays that are based on microbial culture or nucleic acid detection techniques are indirect, time-consuming, and labor-intensive.[25] Contrary to that, bacteria-specific fluorescent probes are designed for direct staining of the bacteria in the sample, enabling fast and sensitive detection of pathogens in complex media. Fluorescent probes could shorten the duration of the assay to minutes and increase its throughputness and cost-effectiveness.

Several types of bacteria-targeting fluorescent molecular probes have been developed. Various pathogen-specific methods of fluorescence labeling have been devised aiming at distinct phenotypic features of the bacterial cell.[26,27] A major group is represented by engineered fluorescent analogs of natural molecular building blocks that can be metabolically incorporated into the bacterial biopolymers and therefore render the exposed cells fluorescent.[28] The most prominent examples are synthetic analogs of sugars and D-amino acids.[29–31] Furthermore, fluorogenic substrates for bacteria-specific enzymes can generate a fluorescence response in the presence of bacteria.[32] Alternatively, fluorescent indicators based on small organic dyes that specifically stain bacteria have been designed.[24,33–36] Finally, bacteria-targeting fluorescent probes have been constructed by conjugating a fluorescence emitter with bacteria-targeting recognition units, such as antibiotics,[37] antibodies,[38,39] aptamers,[40] and cationic antimicrobial peptides (AMPs).[41] The latter ones hold great promise for the development of bacteria-targeting biosensors.[42–44] The use of AMPs as bacteria-recognizing units offers the following advantages. First, tens of thousands of AMPs with distinct selectivity profiles toward different types of bacteria have been described,[45] enabling data-driven selection of an appropriate molecular vector. Second, the binding affinity, selectivity, and stability of the vector can be tuned by chemical modifications of the peptide. In general, recognition units based on AMPs are more robust and amenable to chemical modifications compared with antibodies and aptamers. Third, as many AMPs of practical interest are relatively short, they can be synthesized using solid-phase peptide synthesis (SPPS) and then conjugated to a reporter group using appropriate bioconjugation methods. Combining the broad range of available biocompatible fluorophores gives researchers a great degree of flexibility in designing and customizing peptide-based fluorescence staining reagents. Because of these factors, AMP-derived fluorescent probes have been intensively used for sensing and imaging of bacterial infections.[46–50] At the

same time, the existing fluorescent AMPs display several limitations that need to be addressed. For instance, some fluorophores used in AMP-based reporters exhibit "always-on" fluorescence. Such probes suffer from the non-specific background signal and thus require additional washing steps to remove the unbound fraction from the sample. Another group of AMP-based probes exhibits a fluorogenic behavior, but emit in the green region of spectra.[46] Such optical properties limit the range of applications because of the autofluorescence of biological specimens. Very few fluorogenic AMP-based probes operating in the red spectral region have been described.[47] Here we report a new deep-red fluorogenic bacteria-targeting peptide based on an environmentally sensitive and fluorescent unnatural amino acid covalently incorporated in the amino acid sequence. We show that the internal labeling approach is superior to the commonly used end-labeling, enabling rapid wash-free fluorescence staining of bacteria. The probe is suitable for one-step "add-and-read" staining of both Gram-positive and Gram-negative bacteria by fluorescence microscopy and flow cytometry in buffers and in conditions mimicking bacteriuria.

Results

Design and synthesis of the fluorescent peptides. Numerous AMPs with distinct selectivity profiles have been used in the design of biosensors.[42–44] Among them, ubiquicidin has received much attention as a broad-spectrum bacteria-targeting molecular vector. Ubiquicidin was originally identified in murine macrophages as a cationic AMP of 59 amino acids with a sequence derived from the ribosomal protein S30.[51] Synthetic ubiquicidin and its fragments retain various degrees of antimicrobial activity.[52] Ubiquicidin and its synthetic derivatives non-covalently bind to a wide range of Gram-positive and Gram-negative bacteria, including human pathogens. The binding mechanism is presumably based on the electrostatic interactions between the positively charged side chains of the peptide and the negatively charged bacterial membranes.[53] As a result, ubiquicidin derivatives labeled with fluorescent reporters[46,50,54,55] or radioligands[56–60] have been exploited in the diagnostics and treatment of bacterial infections.[61,62] We hypothesized that ubiquicidin can be converted to a deep-red fluorogenic peptide suitable for one-step wash-free staining of bacteria. To achieve this goal, an appropriate environmentally sensitive fluorophore should be incorporated in ubiquicidin to render the peptide conditionally fluorescent while preserving its binding towards the broad range of bacteria. Nile Red (**NR**, Figure 1a) seemed a fitting fluorophore to accomplish this objective. **NR** is a neutral hydrophobic fluorophore that emits in the red and exhibits an environmentally sensitive fluorescence due to the charge transfer character of the excited state.[63] **NR** displays a significant hypsochromic shift in emission and a strong fluorescence enhancement upon transition from aqueous to hydrophobic environments. The environmental sensitivity makes **NR** a potent probe for biological systems and an attractive reporter moiety for targeted fluorogenic probes.[64] When **NR** is covalently tethered to a ligand, the resulting conjugates exhibit low fluorescence in the free form where the fluorophore is exposed to the aqueous media. At the same time, their fluorescence increases significantly upon binding to the corresponding macromolecular target, where the fluorophore becomes partially screened from the polar environment.[65,66] To incorporate the **NR** fluorophore in ubiquicidin, we used our recently reported **NR**-based fluorescent unnatural amino acid **Alared** (Figure 1a), which has been successfully incorporated in bioactive peptides for the labeling of GPCR in living cells under no-wash conditions.[67] Like the parent fluorophore, **Alared** exhibits polarity-dependent changes in the fluorescence spectra, with an increasing red shift and suppressed fluorescence intensity upon transition from organic solvents to water (Figure 1b).

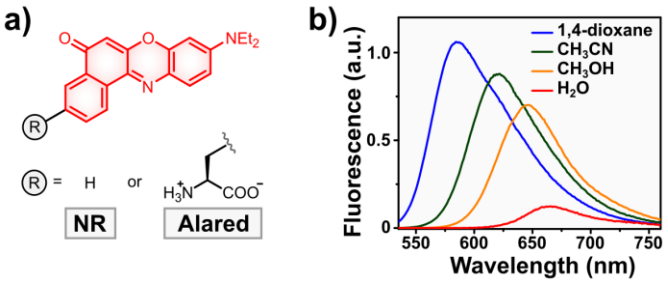

Figure 1. (a) Structure of Nile Red (**NR**) and the fluorescent unnatural amino acid **Alared**[67] used in this study. (b) Fluorescence spectra of free **Alared** in solvents of different polarity.

To obtain bacteria-targeting red fluorogenic probes, we designed two ubiquicidin analogs bearing covalently incorporated **NR** moieties. The peptide design stemmed from the existing literature data on the fluorescence labeling of ubiquicidin. We focused on the truncated version of ubiquicidin named Ubi29-41 composed of thirteen amino acids. Ubi29-41 has received much attention as a vector for infection imaging probes.[46,50,54,55] The fragment containing six positively charged residues was predicted to be disordered in solution (Figure S1). This pointed towards the possibility that the insertion of the unnatural amino acid into the sequence could be tolerated because it did not destabilize any pre-existing secondary structure. We decided to incorporate **Alared** at the Y38 position as it is the only position that contained a natural aromatic amino acid. In addition to the Y38**Alared** mutation, we implemented the norleucine substitution of methionine at position 36, which has been shown to improve the stability of the peptide.[46] The resulting internally labeled peptide was named **UNR-1** (Figure 2a). For comparison, we synthesized an end-labeled version of Ubi29-49 incorporating another derivative of **NR** conjugated at the N-terminus via a minimal flexible linker. The resulting peptide was named **UNR-2** (Figure 2a).

The designed **NR**-labeled peptides were synthesized using a combination of automated and manual solid-phase peptide synthesis following the Fmoc/tBu strategy. The peptides were purified by reverse-phase semi-preparative HPLC and their purity and identity were confirmed by HPLC and HRMS.

Spectroscopic characterization of the fluorescent peptides. **UNR-1** and **UNR-2** each displayed a single fluorescence emission band with maxima around 660–665 nm in aqueous solutions. Both spectra were similar to the emission of the parent fluorophore (**NR**) under identical conditions (Figure 2b). No blue shift was observed in the emission of **UNR-1** compared to **NR**. This observation indicated that within the internally labeled peptide, the fluorophore was fully exposed to the polar aqueous environment, which is in agreement with the predicted unstructured character of this fragment (Figure S1).

Next, we examined the fluorogenicity of the **NR**-labeled peptides. Several factors contribute to the fluorogenic properties of the **NR**-based probes.[65,66] First, the fluorogenic effect arises from the strong increase of fluorescence quantum yield in apolar and aprotic environment (Figure 1b). The second factor contributing to the fluorogenicity of the **NR**-based probes is a substantial positive solvatochromism in absorption spectra, which is seen by a red shift (~ 80 nm) of the absorption maximum of the fluorophore upon transition from apolar solvents to water (Figure S2a). The amplitude of this change in relative absorption depends on the excitation wavelength (Figure S2b). For instance, the relative absorption of **NR** between water and 1,4-dioxane undergoes a 4.2-fold increase at 488 nm, 2.3-fold increase at 520 nm, and 3.5-fold decrease at 561 nm. This solvatochromism is significant enough to contribute to the change in the apparent brightness of the fluorescent probe upon binding to the target. To quantify the combined effect exerted by the changes in absorption and quantum yields, we measured the total fluorescence (fluorescence intensity integrated across the entire emission spectrum) of the parent fluorophore and the labeled peptides under various conditions. We used a small set of organic solvents (DMSO, EtOH, MeOH) and aqueous

solutions (neat water, PBS, and a rich medium for bacterial growth). We performed measurements at three excitation wavelengths that are typically used in fluorescence readers and are compatible with **NR** (488, 520 and 561 nm). The results are presented in Figure 2c. The overall pattern in the data showed that all three compounds fluoresce better in organic solvents than in aqueous solutions. Similar trends were observed for the peptides compared to the free fluorophore, implying that the peptides maintain the fluorogenic character of **NR**. The consistent pattern observed in the data across all experimental conditions suggests that **UNR-1** and **UNR-2** are capable of exhibiting a fluorogenic response upon interaction with macromolecular targets.

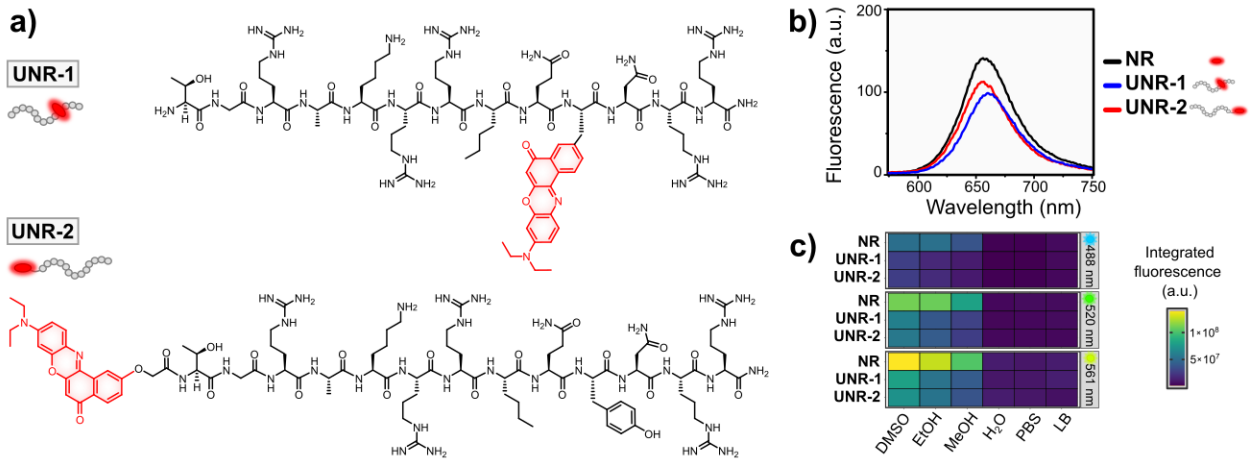

Figure 2. (a) Structure of the Nile Red-labeled fluorescent probes **UNR-1** and **UNR-2** derived from the antimicrobial peptide ubiquicidin. (b) Uncorrected fluorescence spectra of 1 µM solutions of **NR** and the probes **UNR-1** and **UNR-2** in PBS, λ_{ex} = 520 nm. (c) Integrated fluorescence intensity of 1 µM solutions of **NR** and the probes **UNR-1** and **UNR-2** in different organic solvents and aqueous solutions upon excitation at 488 nm, 520 nm, and 561 nm. PBS = phosphate buffered saline; LB = Luria-Bertani medium.

Staining of bacteria by flow cytometry. We examined the possibility of direct staining of live bacteria with the new peptidic probes, using *B. subtilis* and *E. coli* as representative Gram-positive and Gram-negative bacteria, respectively. We shortly incubated live *B. subtilis* and *E. coli* cells in the early logarithmic growth phase with the peptidic probes in LB growth medium and then directly injected bacteria into a flow cytometer without washing out the unbound probe. **NR** was used as a non-specific control. The incubation of the bacteria with both **UNR-1** and **UNR-2** probes resulted in a significant increase in the mean fluorescence intensity upon excitation at 488 nm and fluorescence detection using a red emission filter (Figure 3a). Having confirmed the compatibility of our probes with flow cytometry measurements, we then examined whether the observed mean fluorescence intensity of the bacteria population depended on the concentration of the probe in solution. **UNR-1** stained both *E. coli* and *B. subtilis* in a concentration-dependent manner (Figure 3b). The same tendency was observed for **UNR-2**, albeit with a considerably diminished fluorescence intensity. The non-targeted dye **NR** sufficiently stained the Gram-positive bacteria, whereas only a weak signal was observed for *E. coli*. The flow cytometry data indicate that, among the tested fluorescent labels, **UNR-1** is the most efficient one, as it stains both Gram-positive and Gram-negative cells at reasonable working concentrations (1–10 µM) and short incubation times of several minutes. The concentrations used for staining are significantly lower than the minimal inhibitory concentration (MIC) determined for the non-modified parent peptide Ubi29-41 M36Nle (>64 $\mu g \cdot mL^{-1}$).

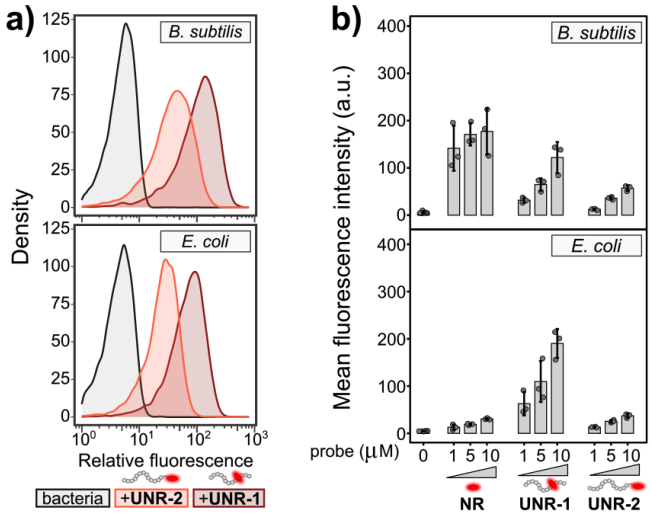

Figure 3. Flow cytometry results and analysis of the cellular localization of **UNR-1**. (a) Flow cytometry analysis of the representative Gram-positive and Gram-negative bacteria incubated with the peptide probes **UNR-1** and **UNR-2**; the labeling was performed by adding the peptides (5 μM) to the bacteria in the early logarithmic phase shortly before measurements; excitation wavelength was 488 nm; fluorescence was recorded using a 680/30 emission filter. (b) Mean fluorescence intensity of the bacteria stained with varying concentrations of **NR** and the **NR**-based peptides; error bars represent standard deviation for $n \geq 3$ biological replicates.

Next, we used flow cytometry to examine the ability of **UNR-1** to stain bacteria that were inactivated by heat or by paraformaldehyde (PFA). The probe sufficiently stained both tested bacteria strains that were inactivated at 90 ºC for 15 min, with overall higher mean fluorescence intensity than for live bacteria (Figure S3). In addition, **UNR-1** stained paraformaldehyde-fixed bacteria, although the results varied depending on the protocol used (Figure S4). Low fluorescence was observed when cells were treated with PFA after labeling, likely due to the dissociation of the probe during washings (Figure S4a). Fixation followed by labeling in phosphate-buffered saline resulted in robustly high staining for both the Gram-positive and Gram-negative bacteria (Figure S4b). The increase in fluorescence cannot be attributed to the change of the buffer alone because the incubation of the probe with live bacteria in PBS gave a significantly lower fluorescence intensity (Figure S4c). Altogether, the results of flow cytometry experiments showed that **UNR-1** is a convenient fluorescent probe to stain both live and dead Gram-negative and Gram-positive bacteria.

Selectivity of the probes. We used fluorescence microscopy to characterize the selectivity of the probes for the bacteria versus the mammalian cells. We incubated live *E. coli* K-12 cells with 1 μM of each probe in the culture growth medium and then imaged them under no-wash conditions. Cells were co-stained with a blue fluorescent DNA-specific dye. Cultured adherent mammalian cells (HEK 293T) were used for comparison. The parent dye (**NR**), which is known to non-selectively stain lipid membranes and other hydrophobic cellular structures in eukaryotic cells, was used as a control. Imaging was performed on a confocal fluorescence microscope using identical excitation and emission settings for each channel. The results are shown in Figure 4. As expected, unconjugated **NR** non-selectively stained the cytoplasmic and internal lipid membranes in HEK 293T cells, including the nuclear membrane. At the same time, the incubation of live *E. coli* with **NR** resulted in a dim diffuse signal that was not associated with the cells, likely due to the poor permeability of the outer membrane of Gram-negative bacteria for exogenous molecules. The inverse staining pattern was observed with the probes **UNR-1** and **UNR-2**. We observed

that both **NR**-labeled peptides stained the bacteria but not the eukaryotic cells. A sufficient level of contrast was observed in the presence of the unbound peptide in solution (no-wash conditions), which was especially prominent with **UNR-1**. These observations supported our initial hypothesis about the possibility of detecting bacteria under no-wash conditions due to the selective binding and the fluorogenic nature of the new probes.

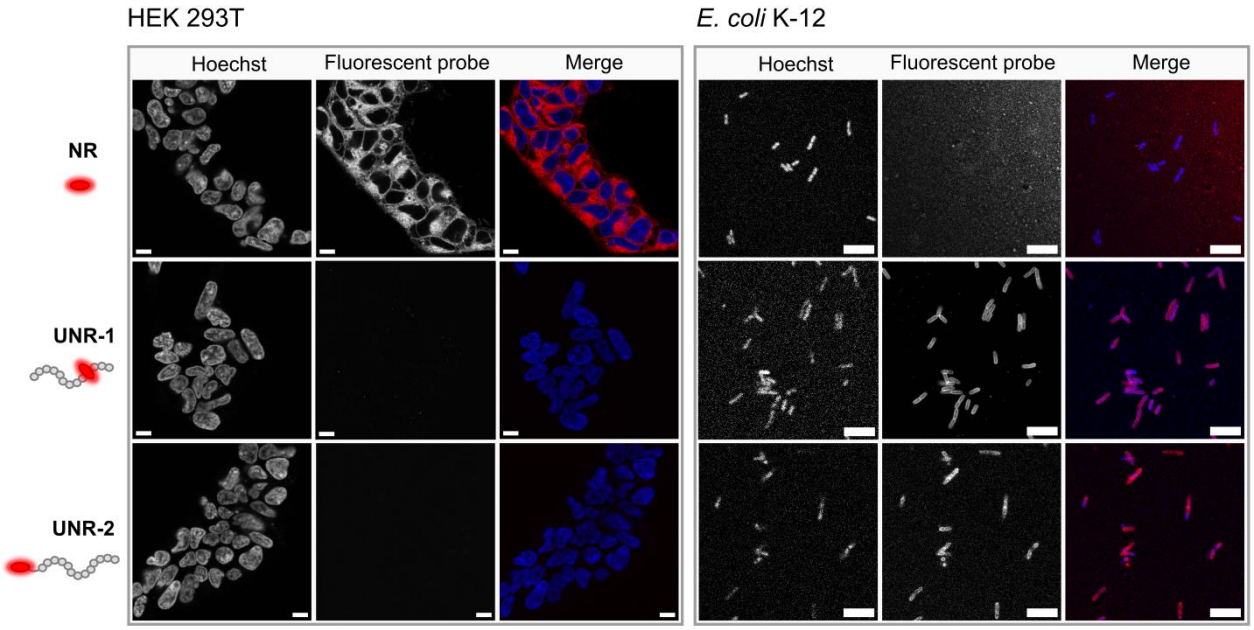

Figure 4. Confocal fluorescence microscopy images of live HEK 293T and *E. coli* K-12 cells stained with 1 μM of **NR** or the peptides **UNR-1** and **UNR-2** under no-wash conditions (λ_{ex} = 561 nm, λ_{em} = 570–730 nm). The cellular DNA was stained with Hoechst 33342 (λ_{ex} = 405 nm, λ_{em} = 430–480 nm). The images in each channel were recorded using identical instrumental settings. Representative images of at least two independent replicates. Scale bars, 10 μm.

Study of the localization of UNR-1 in bacteria. To reveal the localization of **UNR-1** in bacteria, we first tested the compatibility of our probes with super-resolution microscopy. Incubation of live bacteria with **UNR-1** resulted in spontaneous fluorescence blinking observed on the millisecond time scale. The most probable explanation of the "on" events was transient immobilization of the peptide upon binding to the cell in combination with the increased brightness of the bound probe. The "off" events likely occurred due to dissociation of the probe or photobleaching. Analogously to **NR** and its derivatives,[68,69] the blinking behavior of **UNR-1** made the probe compatible with single-molecule localization microscopy (SMLM). The on/off switching of **UNR-1** enabled recording of individual molecular localizations over a period of time followed by reconstructing a sub-diffraction image of the labeled cell. Using an SMLM setup (Figure 5a), we imaged *E. coli* and *B. subtilis* as representative Gram-positive and Gram-negative bacteria (Figures 5b and 5c). The observed characteristic structures indicated that the probe was bound to the outer layers of the cell.

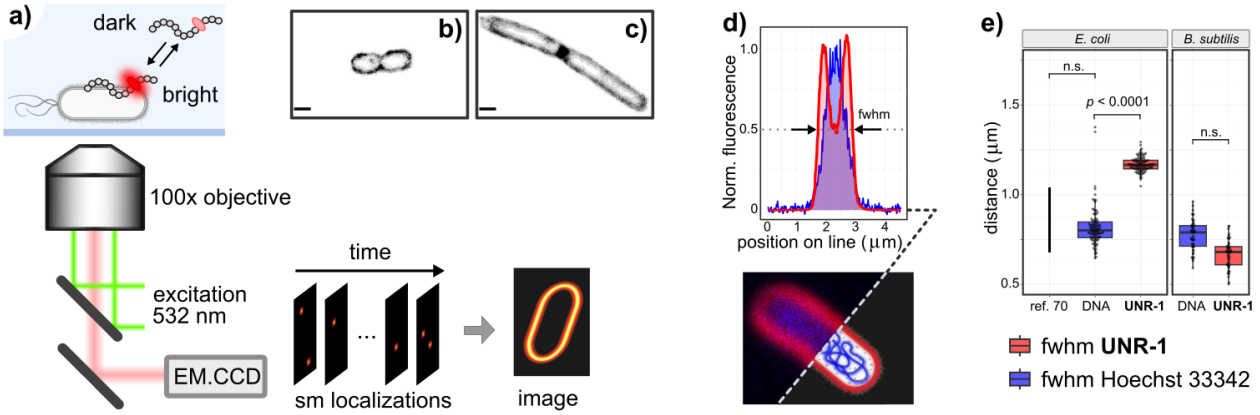

Figure 5. (a) Single-molecule localization microscopy with **UNR-1**. (b) and (c) Super-resolution fluorescence microscopy images of **UNR-1**-labeled *E. coli* (b) and *B. subtilis* (c); scale bars, 1 µm. (d) Normalized fluorescence intensity profiles obtained from two-color confocal fluorescence images of bacteria co-labeled with **UNR-1** (red) and Hoechst 33342 (blue); fwhm = full width at half-maximum. (e) Comparison of the fwhm values of the red and blue fluorescence intensity profiles for *E. coli* and *B. subtilis* (N >50 cells, one-tailed Wilcoxon rank sum test, n.s. = non-significant); reference diameter of *E. coli* cells was taken from ref.[70]

We performed an additional experiment to more specifically characterize the localization of the probe in the Gram-negative and positive bacteria. We hypothesized that insights into the localization of the probe with respect to the bacterial plasma membrane could be obtained from the analysis of cells co-labeled with **UNR-1** and a DNA-specific fluorescent dye. Because the cellular DNA is contained within the uncompartmentalized cytoplasm, the DNA-associated fluorescence signal should to mark the internal volume of the cell. Comparing the dimensions of the DNA and **UNR-1** fluorescence blobs in diffraction-limited images, one can conclude whether the probe is localized in proximity to the cytoplasm, at the plasma membrane, or the periphery of the cell bound to the outer layers of the cell wall. To analyze the probe location, we perpendicularly sectioned the bicolor confocal fluorescence images of bacteria and measured the full width at half-maximum (fwhm) of the normalized fluorescence signal for each fluorophore (Figure 5d). For *E. coli*, the measured mean value of fwhm for the DNA stain was ~ 0.79 µm. This distribution of fwhm values matched well the diameter range of the bacterium that has been measured by electron microscopy under different growth conditions (Figure 5e).[70] Meanwhile, the fwhm of the red signal generated by the probe **UNR-1** was significantly larger, with a mean value of ~ 1.17 µm (Figure 5e). On the contrary, for the Gram-positive bacteria *B. subtilis*, the signal of **UNR-1** did not exceed significantly the fwhm of the signal associated with DNA. This indicated that, in the case of *E. coli*, the peptide probe is most likely localized at the outer layers of the cell wall, such as the outer membrane. In the case of *B. subtilis* the probe resided in close proximity to the cytoplasm, most likely at the plasma membrane.

Detection of bacteria in urine. Bacteriuria is the presence of bacteria in urine at a level of $\geq 10^5$ bacteria·mL^{-1}.[71] In most cases, bacteriuria is caused by *E. coli*. Fast detection of bacteria in urine is important for the diagnosis of urinary tract infections (UTIs) and other disorders. In a proof-of-concept experiment, we tested the ability of **UNR-1** to directly stain *E. coli* in urine samples. In one case, sterile urine was freshly inoculated with *E. coli* previously grown in LB. In a second case, *E. coli* was grown in sterile urine overnight. Both samples were stained with 5 µM **UNR-1** and analyzed by flow cytometry. In both cases bacteria were robustly stained with **UNR-1** (Figure S5), proving its utility as a probe for the detection of bacteria in biological liquids.

Discussion

Fluorogenic peptide-based probes for bacterial detection emitting in the red and near-infrared regions of the visible spectrum are of considerable practical interest because they could offer a combination of high affinity to the target, high signal-to-noise ratio, and fast response. This combination is highly beneficial in the analysis of biological samples with high blue-green autofluorescence. While fluorescent unnatural amino acids appear to be ideal building blocks for labeling of peptides, most of them fluoresce in the blue and green regions of the spectrum.[72,73] The amino acid **Alared** employed as a reporter in this work is a rare example of compact fluorescent unnatural amino acid with green-yellow excitation and deep red emission. The emission maxima of **Alared** in aqueous solutions (~ 665 nm) is higher than the previously reported fluorescent amino acids such as Phe(pMP-BODIPY)-OH (625 nm),[74] benzodiazole-derived amino acids (up to 652 nm),[75] and Rf490DL (660 nm).[76] Moreover, the environmental sensitivity of **Alared** and its compatibility with the conditions of Fmoc/tBu solid-phase peptide synthesis makes this amino acid a promising building block in the design of peptide-based fluorescent probes.

The ability of our probes to fluorescently stain bacteria and their selectivity over mammalian cells have corroborated the potency of ubiquicidin as a bacteria-targeting molecular vector. An interesting finding emerged from the direct comparison of the end-labeled and backbone-labeled peptidic probes **UNR-2** and **UNR-1**, respectively. Unexpectedly, the backbone-labeled probe **UNR-1** gave a fewfold higher signal than **UNR-2** in flow cytometry both for Gram-positive and Gram-negative bacteria. Because the end-labeled probes have been explored more intensively than internally labeled probes, our results suggest that the backbone-labeled design deserves more attention from the community of developers of fluorescent probes.

The capacity of the probe to stain bacteria *in situ* without washing both in a growth medium and in buffers opens many avenues for future practical applications. For instance, **UNR-1** could be used for rapid detection of bacteria in biological and environmental samples using fluorescence microscopy. If combined with an appropriate enrichment method and fluorescence readout device, the probe could be used for the detection of low levels of bacterial pathogens in medical laboratory diagnostics. Unlike the fluorescence staining methods based on metabolic labeling of bacterial polymers, **UNR-1** does not rely on bacterial enzymes. Therefore, this new probe could find applications in staining and detection of viable but nonculturable (VBNC) bacteria, metabolically dormant persister cells, bacterial spores, etc. Finally, the probe could be used for fluorescence staining of bacteria-derived nanostructures, such as various types of bacterial outer membrane vesicles (OMVs). Further research in these directions is ongoing in our laboratories.

Associated content

Supporting Information

Additional chart, Figures S1–S5, synthesis and characterization of the peptides, experimental details for spectroscopy, microscopy, flow cytometry and data analysis.

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Notes

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