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Porphyrin-modified antimicrobial peptide indicators for detection of bacteria

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ABSTRACT

This study demonstrates the potential of porphyrin modified antimicrobial peptides for indication of bacterial targets on the basis of changes in the spectrophotometric characteristics of the construct. Detection is a result of changes in the structure of the antimicrobial peptide upon target binding. Those constructs comprised of peptides that offer little or no change in conformation upon interaction with bacterial cells demonstrated negligible changes in absorbance and fluorescence when challenged using *Escherichia coli* or *Bacillus cereus*. CD analysis confirms the presence/absence of conformational changes in the porphyrin-peptide constructs. Differing spectrophotometric responses were observed for constructs utilizing different peptides. The incorporation of metals into the porphyrin component of the constructs was shown to alter their spectrophotometric characteristics as well as the resulting absorbance and fluorescence changes noted upon interaction with a target. The described constructs offer the potential to enable a new type of biosensing approach in which the porphyrin-peptide indicators offer both target recognition and optical transduction, requiring no additional reagents.

1. Introduction

Antimicrobial peptides (AMPs) are a group of biomolecules that have evolved to recognize and kill target microbes by binding to and disrupting cell membranes. Several unique characteristics of AMPs make them attractive alternatives to antibodies for detection of microbial biothreats: resistance to proteases; stability to environmental extremes; and high affinity, overlapping (but not identical) binding interactions with microbial membranes and membrane components. Arrays of AMPs have been used to detect and classify microbial pathogens with similar sensitivity to antibody-based assays; their broad-spectrum binding activities also provide the potential for detection of unknown microbes [11,12,16,18,27]. In previous studies, surface-immobilized AMPs mediated target binding, and an additional "tracer" (e.g., labeled antibody or non-specific dye) was required for signal transduction. This constraint increases the number of reagents required and the overall complexity of the assay. Development of an AMP-based material that is capable of both target recognition and signal generation without addition reagents or processing steps is highly desirable. This type of construct would provide greatly enhanced potential for application of AMP-based detection techniques in autonomous and distributed sensing platforms.

A number of publications report use of porphyrin-peptide conjugates for targeting and photodestruction of cells [3–5,7,10,13,24]. In these studies, the antimicrobial peptide domain is used to interact with the appropriate cell (cancer cell, Gram-negative bacterial pathogen), while the porphyrin moiety is used as a source of reactive oxygen species upon illumination [21]. Porphyrins are large macrocyclic compounds with strong absorbance and fluorescence characteristics. They have been applied in a wide variety of detection approaches due to the sensitivity of those characteristics to their immediate environment. Spectrophotometric and binding characteristics can be altered through modification of the porphyrin structure. Several reports have described modifications using single amino acids or dipeptides [2,29,33]. Binding of proteins by these porphyrin derivatives resulted in changes to their fluorescence characteristics, and arrays of the constructs were applied to discrimination of proteins. Modification of the periphery of a porphyrin using cytosine was similarly applied to detection of guanine [6]. Other works have shown that porphyrins can be used to report

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conformational changes in enzymes upon substrate binding when the porphyrin-enzyme interaction results in competitive or mixed-type enzymatic inhibition [30,31].

This study sought to demonstrate the potential for antimicrobial peptides modified using porphyrins in indication of the presence of bacterial cells. The goal was development of constructs providing an avenue for achieving reagentless detection and classification of bacterial targets. Sensing in this case would utilize changes in the local environment of a covalently attached porphyrin resulting from conformational changes in the antimicrobial peptide. While others have proposed application of porphyrin-peptide conjugates as imaging agents (e.g., [13]), this approach would provide the potential for use of an array of peptide-porphyrin conjugates in detection of bacteria with broad classification of the detected cells based on the differential changes in the spectrophotometric characteristics of the porphyrin-peptide conjugates. Here, synthesis and characterization of a set of four porphyrin-AMP constructs is presented. Their utility with regard to the potential for indication of bacterial targets is discussed.

2. Methods

5-Mono(4-carboxyphenyl)-10, 15, 20-triphenyl porphine (C_1TPP) was obtained from Frontier Scientific (Logan, UT). Vanadium (III) bromide, zinc chloride, cobalt (II) chloride, and dimethylsufloxide (DMSO) were obtained from Sigma-Aldrich (St. Louis, MO). 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), N-hydroxysuccinimidyl ester (NHS) and sulfo-NHS were purchased from Pierce Thermo Scientific (Rockland, IL). Antimicrobial peptides indolicidin (Ind), bactenecin (Bac), and cecropin A (1–8)-melittin (1–18) hybrid peptide (CeMe) were purchased from American Peptide Company (Sunnyvale, CA); polymyxin E (PME) was obtained from Sigma-Aldrich. Sequences are provided in Table 1.

Direct covalent attachment of C_1TPP to the above peptides was accomplished under anhydrous conditions with carbodiimide-mediated coupling. Stock solutions of C_1TPP, EDC, NHS, bactenecin, and indolicidin were prepared in absolute ethanol prior to mixing; as neither PME nor CeMe is highly soluble in absolute ethanol, stock solutions of these peptides were prepared in 4:1 (*v*/v) ethanol:acetonitrile. The composition of the reaction mixtures (molar equivalents) were as follows: 1 peptide: 1.1 C_1TPP: 1.2 EDC: 1 NHS. After completion of the coupling reaction (≫2 h), reaction mixtures were diluted with water and dialyzed (1000 molecular weight cutoff) exhaustively against water and phosphate-buffered saline (PBS). Construct concentrations are estimated based on the initial concentration of AMP and the total final volume of the preparation. Metal variants were prepared through incubation of porphyrin-AMP constructs (25 μM) with metal salts (vanadium (III) bromide; zinc chloride; cobalt (II) chloride; 50 μM) in aqueous solution [8]. The solutions were thoroughly mixed and heated to 60 °C for 3 h before storage at 4 °C for at least 48 h. Metal incorporation was evaluated based on changes in absorbance and fluorescence characteristics. Construct names are abbreviated to indicate the metal, porphyrin, and AMP used; for example, CoC_1-Ind is the cobalt variant of C_1TPP conjugated to the indolicidin peptide.

The bacterial targets for binding studies, *Escherichia coli* (XL1 blue) and *Bacillus cereus* (ATCC 10987), were grown to mid-log in Luria (37 °C) or tryptic soy broth (30 °C), respectively, before harvesting by centrifugation at 1200 ×*g* for 10 min (4 °C). Cell pellets were washed twice with phosphate-buffered saline (PBS), pH 7.4 and resuspended in 1/5 original volume of PBS. Cell numbers (in PBS) were then counted by flow cytometry (Accuri C6). Cell suspensions not used immediately were diluted with an equal volume of 60% glycerol in PBS before storage at −20 °C. Prior to analysis, cells were diluted in PBS to the appropriate concentrations.

A Tecan XSafire microtiter plate reader was used to measure the absorbance and fluorescence of the porphyrin-AMP constructs in the presence and absence of bacterial targets. Absorbance was measured from 360 to 800 nm in steps of 2 nm. Fluorescence emission spectra were collected from 500 to 800 nm (2 nm steps) using 415 nm excitation while fluorescence excitation spectra were collected from 385 to 619 nm (2 nm steps) at 730 nm emission. In both cases, a gain of 160 was applied with 50 flashes at 400 Hz, and an integration time of 20 μs was employed. All experiments were conducted in 15% DMSO in order to ensure a homogeneous solution; porphyrin-AMP constructs have low water solubility due to the hydrophobicity of the porphyrin utilized and inherent solubilities of the AMPs. Cell concentrations ranging from 107 to 103 cells/mL were employed. Indicator concentrations were varied from 12 to 0.1 μM. In all cases, difference spectra were calculated as the point-by-point subtraction of indicator only spectra from spectra collected for the indicator in the presence of the target.

Fluorescence spectra for cell pellets utilized a total initial volume of 765 μL with 8 μM indicator and varying target cell concentrations in an Eppendorf tube (1.5 mL). As above, all experiments were conducted in 15% DMSO. The fluorescence of the initial solution was measured before centrifuging at 7500 rpm for 10 min. Supernatant was then removed (665 μL), and the remaining solution and pellet were mixed thoroughly to resuspend components. The fluorescence of both the resulting supernatant and the resuspended pellet were collected using the microtiter plate protocol described above.

CD experiments were carried out using a Jasco J-815 circular dichroism spectrometer (Jasco Inc., Easton, MD). An estimated peptide concentration of ~10 μM was used for each CD measurement. CD spectra were recorded in phosphate buffer (PB), pH 7.0 in the presence or absence of 25 mM sodium dodecylsulfate (SDS; critical micelle concentration of SDS ~8.0 mM), as indicated. The wavelength scan was completed from 190 to 300 nm in a thermally controlled (20 °C) quartz cell having a 0.5 cm path length. Each CD spectrum was the average of three scans collected at a scan rate of 50 nm/min, using a data pitch of 1 nm, digital integration time (D.I.T) of 8 s, band width of 1 nm, and a scan speed of 50 nm per minute. The background spectra (i.e., PB buffer ±25 mM SDS in the cuvette) were measured first, followed by that of the peptide solution. Subtraction of the background (solution) spectrum from that of the peptide solution yielded the spectrum of the peptide in the absence or presence of SDS.

Obtaining accurate concentrations of certain peptides is difficult, but reliable structural content calculations can be performed without knowing the exact concentrations of peptide by *g*-factor analysis [15]. The dimensionless *g*-factor is independent of path length, concentration, amino acid content, and molecular weight and is calculated when the same sample and cell are used for both CD and absorption measurements. A *g*-factor spectrum is calculated by dividing the differential absorbance of left- and right-handed circularly polarized light (A_l and A_r,

Table 1
Antimicrobial peptide characteristics.

Peptide	Sequence	Structure in solution	Structure on interaction	Ref
Indolicidin	ILPWKWPWWPWRR-NH_2	Unstructured	Extended boat	
Cercropin A-melittin hybrid	KWKLFKKIGIGAVLKVLTTGLPALIS-NH_2	Unstructured or β-sheet	Amphipathic α-helix	[1]
Bactenecin	RLCRIVVIRVCR; cyclized via disulfide bridge	β-structured	Minimal change	[32]
Polymyxin E	Fatty acyl chain-BTBBBLLBBT, B = diaminobutyrate; cyclized via the side chain of B4	Cyclized peptide possessing a fatty acyl tail	Minimal, loss of backbone turns	[20]