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Cationic Antimicrobial Peptides (AMPs): Thermodynamic Characterization of Peptide–Lipid Interactions and Biological Efficacy of Surface-Tethered Peptides

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The worldwide emergence of bacterial superbugs, and the limited number of effective antibiotic against them, has become an unresolved public catastrophe. According to a report from the World Health Organization in 2014, the resistance rate in a common bacterial pathogen such as *Escherichia coli* is high enough to limit the efficacy of currently available oral treatment options for patients with urinary tract infections; if cases where bacteria adsorb and form biofilms on implant materials, the treatment options are even more limited. Thus, the development of novel antibiotics for oral administration and as coatings for biomaterial surfaces represents an important and pressing research goal.

A wide variety of organisms produce antimicrobial peptides (AMPs) as part of their defense system. The main site of action of the mostly positively charged and amphipathic AMPs is the membrane of the target cells. AMPs accumulate on the surface of the bacterial cell membrane and insert into the lipid matrix. This leads to disruption of the barrier function of the membranes and cell death. The AMPs bacterial selectivity is highly dependent upon the peptide structural motifs as well as the differences in organization of the cell membranes. Unlike the cholesterol-rich electrically neutral lipid membrane of eukaryotic cells, responsible for low peptide affinity and insertion, the bacterial cytoplasmic membrane possesses negatively charged phospholipids that, together with additional cellular envelope features such as the lipopolysaccharide (LPS) and peptidoglycans in Gram-negative and Gram-positive bacteria, respectively, represent ideal targets for cationic AMPs binding. This mode of action of AMPs could represent a basis for developing a novel class of antibiotics to counteract the issue of bacterial resistance.

In this doctoral research, the structural basis of activity of short cyclic AMPs rich in arginine and tryptophan residues, as promising anti-*E. coli* candidates, was studied to understand membrane constituents and the peptide structural motif important for selectivity against Gram-negative bacteria. In addition, optimized chemical strategies were developed for tethering AMPs with different modes of action on polyethylene glycolylated (PEGylated) resin beads as a model solid surface. The biological activity of the surface-bound AMPs against bacteria was investigated and compared with the activity of the corresponding free peptides in solution. In particular, we sought to understand how a) the physical properties of the resin, such as the spacer length between the solid matrix and the AMP, as well as the capacity of the functional groups on the surface, and b) the tethering at different positions (peptide termini and side chain) affect the activities of the tethered AMPs.

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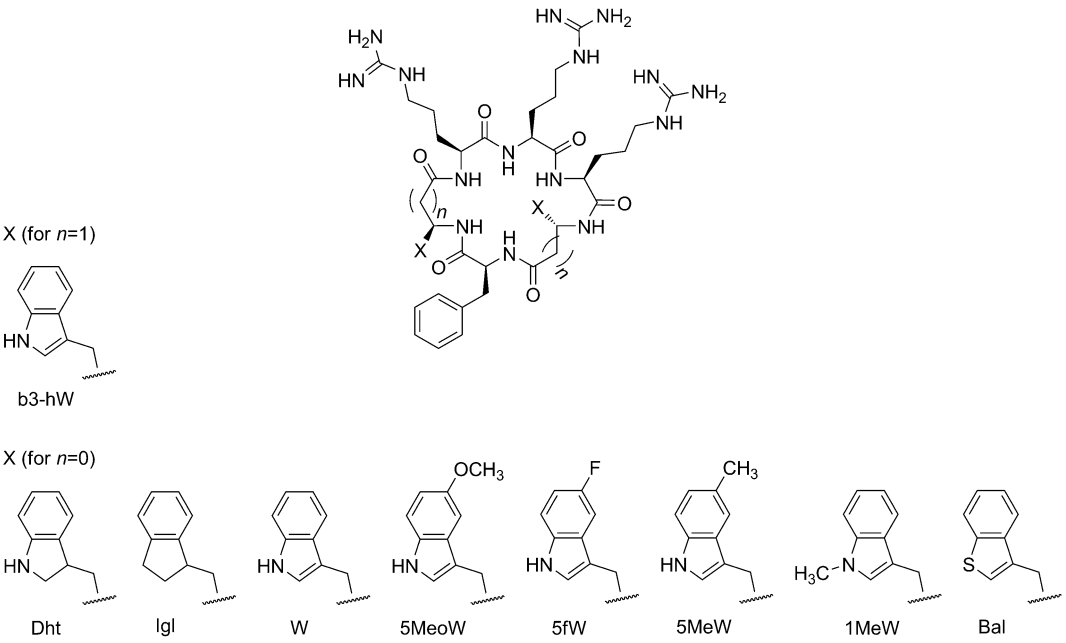
Structural basis of anti-*E. coli* activity of c-WFW

cyclo-RRRWFW (c-WFW) shows excellent activities against various strains of bacteria (in particular Gram-negative such as *E. coli*) compared with its linear analogue, Ac-RRRWFW (Ac-WFW). The small size and cyclic structure imparts good metabolic stability against proteases, therefore making c-WFW a promising lead candidate for further activity optimization. The roles of both the arginine and tryptophan residues in biological and bilayer permeabilizing activities are described for several peptide variants. The susceptibility of Gram-negative bacteria to c-WFW was proposed to be associated with factors that facilitate the transport of the peptide across the LPS. c-WFW is most active against the smooth LPS strain (wild type), while reduction of sugar chains in mutated LPS strain (rough type) distinctly decreases the antimicrobial effect. At the lipid membrane level, the electrostatic attraction favors binding between arginine residues in the cyclic peptide and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC)/1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-(1′-*rac*-glycerol) sodium salt (POPG) bilayers (modelling the charge properties of the cytoplasmic membranes of red blood cells and bacteria), and tryptophan residues have high propensity to insert into the membrane and partition near the membrane water interface.

While the significant role of tryptophan residues in many LPS-binding motifs of AMPs is known, studies on the structural motifs of c-WFW in particular from the point of selective interactions between the aromatic side chain of tryptophan residues and distinct regions of LPS, which are important for its anti-*E. coli* activity, needed to be done. For this, a series of c-WFW analogues in which tryptophan residues in the aromatic WFW cluster were substituted with non-natural analogues was synthesized (Scheme 1). These non-natural residues are characterized by altered hydrophobicity, variable dipole and quadrupole moments, modified hydrogen-bonding ability, and changed amphipathicity or enhanced ring size.

The antimicrobial and hemolytic activities of the peptides were evaluated against *E. coli*, *Bacillus subtilis* and red blood cells (Figure 1). The peptide ability to permeabilize the inner and outer membrane of *E. coli* was also investigated. Isothermal titration calorimetric (ITC) studies were carried out to provide information on peptide interaction with POPC and POPC/POPG bilayers, and with lipid A, rough-LPS and smooth-LPS incorporated in POPC bilayers (as models of the outer membrane of Gram-negative bacteria). The results from this study showed that peptide hydrophobicity and backbone constraints are the crucial determinants of biological activity. The different susceptibilities of *E. coli* and



Scheme 1. General structure of the cyclic peptides with the positions for desired modifications, and the chemical structures of tryptophan (W) analogues used in this study. Abbreviations: (γ*S*/γ*R*)-dihydrotryptophan (Dht), (α*S*)-(2-indanyl)glycine (Igl), 5-methoxy-L-tryptophan (5MeoW), 5-fluoro-L-tryptophan (5fW), 5-methyl-D,L-tryptophan (5MeW), 1-methyl-L-tryptophan (1MeW), and β-(benzothien-3-yl)-L-alanine (Bal).

B. subtilis could be explained by differences in the negative surface charge of the plasma membranes (Figure 1 a). Additionally, interaction with the outer membrane LPS of *E. coli* is responsible for the complex activity pattern. The peptides permeabilized the outer membrane according to their anti-*E. coli* activity spectrum, suggesting LPS as the crucial modulator of activity. Low hydrophobicity and conformational flexibility of peptides decreases their affinity for the lipid layers (Figure 2). In the presence of lipid A, rough-LPS and smooth-LPS, the dominant role of hydrophobicity decreased among the cyclic peptides with no influence on the weak affinity of the highly flexible linear Ac-WFW. The higher hydrophobic partition coefficients for the peptide interaction with POPC/smooth-LPS compared with POPC/rough-LPS lipid bilayers underlined the modulating effects of the *O*-antigen and the oligosaccharides in the outer core of LPS in the peptide activity and transport across the *E. coli* outer wall.

Despite promising properties and many efforts to develop peptidic antibiotics, susceptibility to proteolysis, low bioavailability, and possible immunogenicity, as well as the costs of the synthesis, have restricted the therapeutic application of AMP thus far. However, the present study on cyclo-RRRWFW opens the door of hope to tackle these obstacles.

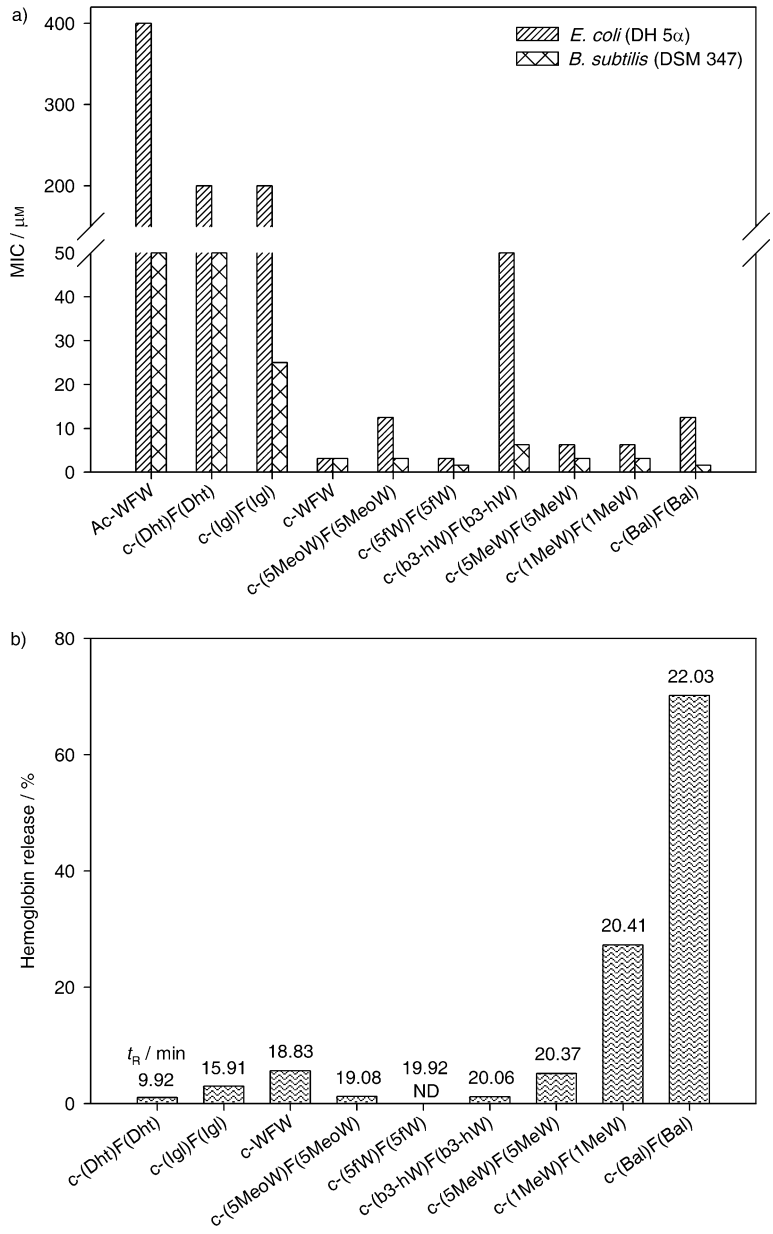


Figure 1. a) Antimicrobial and b) hemolytic activities of cyclic peptide derivatives. MIC values are the minimal concentration of peptide required to inhibit bacterial growth. The hemolytic activity was determined at a peptide concentration of 200 μM; note, c-(5fW)F(5fW) was not evaluated. The peptides were nontoxic at their MIC values. Values shown in panel b represent the HPLC retention time (t_R). ND: not determined.

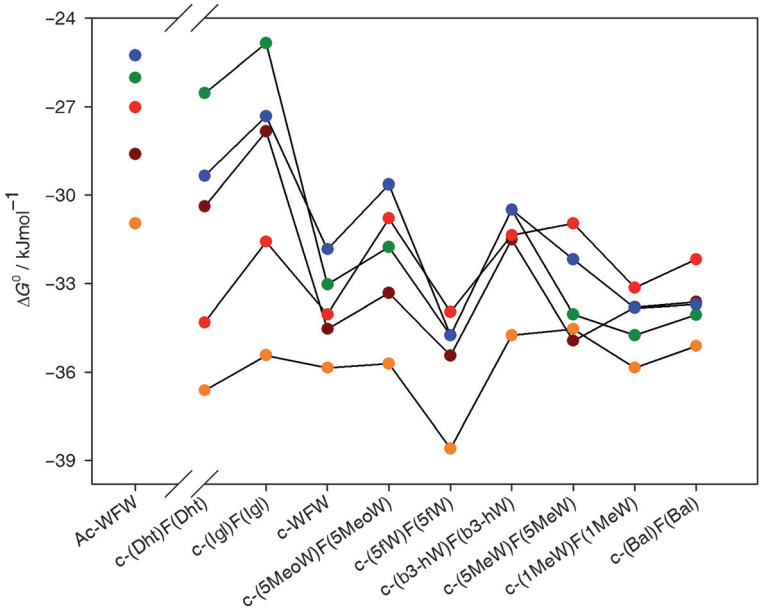


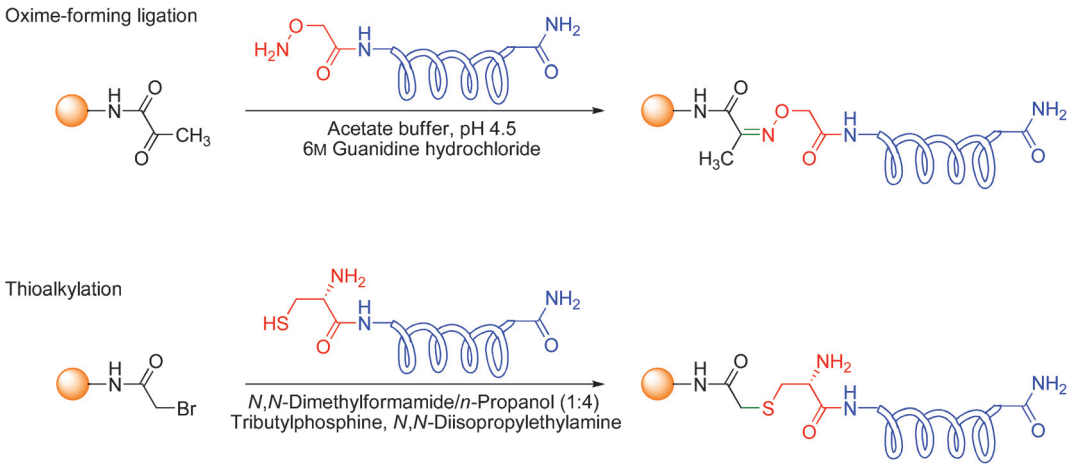
Figure 2. ΔG° values for the binding of cyclic peptide variants to POPC (green), POPC/POPG (3:1) (brown), POPC/lipid A (12:1) (blue), POPC/rough-LPS (12:1) (red), POPC/smooth-LPS (12:1) (yellow) small unilamellar vesicles. Ratios are mol/mol. The binding parameters were calculated by taking a surface partition equilibrium model combined with the Gouy–Chapman theory.

Bioactivities of tethered AMPs

Infections associated with implant materials, especially those caused by bacterial and fungal superbugs, are of increasing concern in modern medicine. Antimicrobial materials prevent microbial infections in a wide variety of medical and industrial settings. To obtain biocidal effect without releasing biocides into the environment, antimicrobial species can be irreversibly coupled to material surfaces. Examples include synthetic polymers coupled covalently to AMPs. However, little is known of the factors determining the antibacterial and anti-inflammatory activities as well as the issues of biocompatibility of these materials and understandings of their interactions with human tissues and cells.

In the second part of the doctoral research, the site-specific covalent immobilization of highly active AMPs was investigated. AMPs studied included two membrane-permeabilizing amphipathic model peptides with a "carpet-like" mode of action: KLAL and magainin 2 amide-derived MK5E peptide; pore-forming melittin (MEL); buforin 2 (BUF2) which binds to intracellular nucleic acids; and tritrpticin (TP) which was described to be membrane-lytic and have intracellular targets. Oxime-forming ligation and thioalkylation strategies were used for coupling of the peptides via the C terminus, N terminus and side chains to resin beads as model surfaces (Scheme 2). The influence of resin parameters, such as length and density of the PEG spacers available for peptide conjugation and the role of the position of the peptide tethering, was analyzed by determining the antimicrobial activities of the tethered peptides.

The antimicrobial activities of free soluble and tethered peptides against *E. coli* and *B. subtilis* represent the major findings of this work. Specifically, the distance between the solid surface and the active sequences was identified as a critical parameter for peptide activity. The AMPs effectiveness decreased with decreasing spacer length, regardless of the amount of peptide on the surface. Immobilization of membrane-permeabilizing sequences was found to be most suitable for the generation of antimicrobial surfaces. Immobilization did not influence the activity pattern and conserved the peptide membrane-permeabilizing mode of action. Tethered AMPs showed anti-*E. coli* and anti-*B. subtilis* activities at millimolar concentrations compared with the micromolar concentrations of the free soluble peptides (Figure 3). The positioning of the peptides within the bacterial membrane, which is determined by the distribution of hydrophobic and charged residues in the sequence, has to be taken into account. In order to conserve high activity, tethering should occur at a position far away from the hydrophobic domain (c.f., activity of MEL tethered at the C and N terminus; Figure 3). In contrast, the activity of sequences with a uniform or symmetric distribution of hydrophobic and positively charged amino acids, for example, KLAL, MK5E, and TP, is less dependent on the tethering position. Membrane-translocating



Scheme 2. Chemical strategies for tethering AMPs. To obtain ketone and bromoacetic acid functionalized resin for oxime-forming ligation (top) and thiolakylation (bottom) strategies, respectively, the beads should firstly be treated with pyruvic acid and 2-bromoacetic acid.

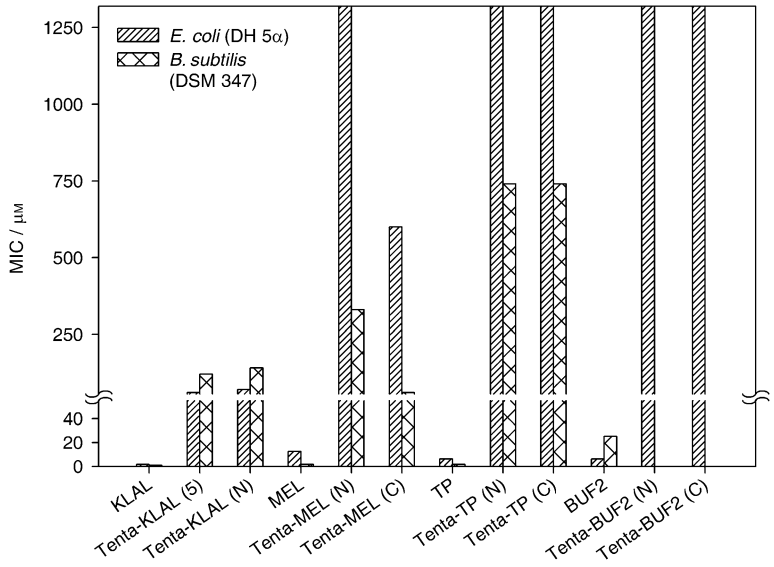


Figure 3. Antimicrobial activities of free soluble and tethered AMPs. Number and letters in parentheses indicate the position of the peptide amino acid side chain or terminus at which immobilization occurred. Anti-*B. subtilis* activities of tethered BUF2 were not determined.

peptides such as BUF2 were inactivated by immobilization. A comparison of the activities of soluble AMPs and peptides tethered at variable positions might also be helpful in gaining insight into the membrane selectivity of peptides.

Keywords: antimicrobial peptides · immobilization · liposomes · materials · thermodynamics

Publications arising from this work:

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