Self-Assembling Antimicrobial Peptides with Intrinsic UV-Visible Spectral Fluorescence upon Single Amino Acid Substitution from Arginine to Citrulline

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Introduction

The molecular assembly of short peptides into ordered structures is a promising approach to developing bio-nanomaterials with diverse applications in drug delivery, electronics, and optical engineering. Intrinsic fluorescence of peptide aggregates is typically associated with the delocalization of electron densities in hydrogen bonding networks and dipolar coupling of aromatic amino acids. Moreover, fluorescence intensity in peptides is related to changes in amyloid morphology, suggesting a link to secondary structure.[1-2] Because of their ease of tuning secondary structure, shorter peptides (≥10 amino acids) are preferable for fabricating ordered intrinsically fluorescent nanostructures.[3]

In this study, we report a distinct example of peptide intrinsic fluorescence observed during the investigation of the influence of hydrophobicity and net charge on peptide self-assembly and antimicrobial activity of β-sheet-forming macrocyclic peptides. Macrocyclic disulfide **1** (*c*-[H-CIVIRFKFRC-NH_2]) was created based on the structure of chicken Angiogenin-4 and shown to adopt a predominant β-sheet conformer, which formed non-fluorescent amorphous aggregates exhibiting antibacterial properties in aqueous media.[4] **Replacement of a single arginine (Arg) residue with citrulline (Cit) in the peptide macrocycle added the hydrogen-bonding of urea, causing a morphological change from spherical non-fluorescent particles to fluorescent rods and fibers of larger size.**

Table 1. Peptide macrocycles and their key parameters.

Name	Peptide Sequence	Net Charge	Experimental M.W. (g/mol)	CMC* (μM)	Particle size (nm)	Zeta Potential (mV)	% Self Assembly after 8 days
1	*c*-[H-CIVIRFKFRC-NH_2]	+3	1282.72	4.8	274 ± 62	+16.8 ± 0.6	44.1
[V^2]-**1** (**2**)	*c*-[H-C**V**VIRFKFRC-NH_2]	+3	1268.70	4.7	282 ± 45	+14.2 ± 1.1	38.7
[V^4]-**1** (**3**)	*c*-[H-CIV**V**RFKFRC-NH_2]	+3	1268.70	4.6	256 ± 89	+16.3 ± 0.8	19.7
[Cit^5]-**1** (**4**)	*c*-[H-CIVI**X**FKFRC-NH_2]	+2	1283.69	1.2	428 ± 96	+5.4 ± 2.3	85.2
[Cit^9]-**1** (**5**)	*c*-[HCIVIRFKF**X**C-NH_2]	+2	1283.70	0.8	685 ± 82	+6.2 ± 0.2	91.3

***CMC :** Critical micelle concentration

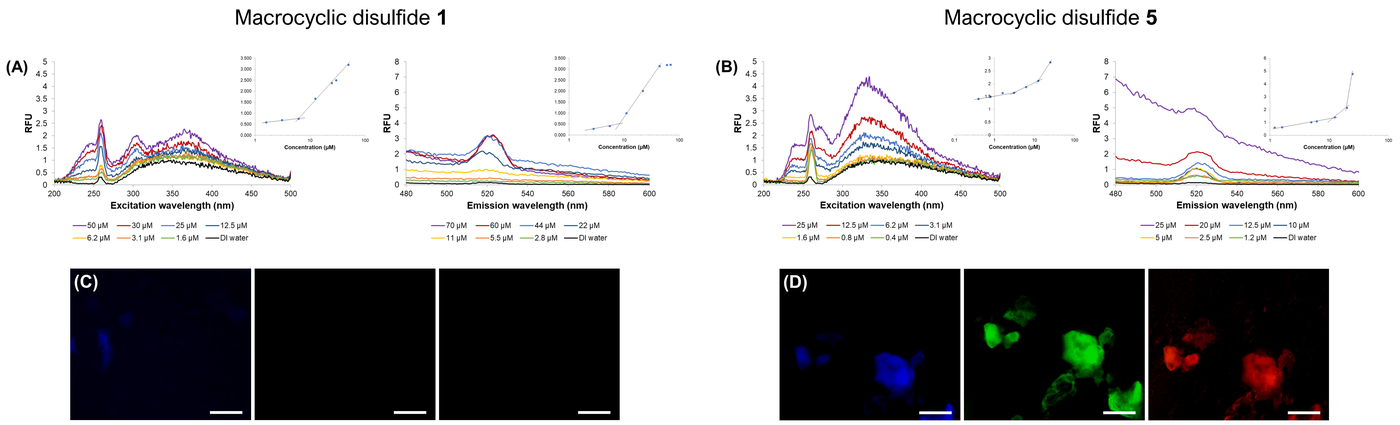

Figure 1. Concentration-dependent fluorescence excitation and emission spectra of (A) **1** and (B) **5** at emission and excitation wavelengths of 520 nm and 260 nm, respectively. **Insets:** plots of fluorescence intensity as a function of peptide concentration (logarithmic scale). Fluorescence microscopy images of peptide (C) **1** and (D) **5** using **DAPI** (left), **FITC** (center), and **TRITC** (right) filter sets. (Scale bar = 50 μm)

Table 2. Assignment of secondary structures present in peptide macrocycles.

Amide I position (cm^{-1})	Assignment	1	2	3	4	5
1611-1616	Aggregated β-strand / Extended strand		✓			✓
1621-1636	β-strand	✓	✓	✓	✓	✓
1653-1659	α-helix					✓
1666-1673	β-turn	✓	✓	✓	✓	✓
1689-1694	Antiparallel β-strand	✓	✓	✓	✓	✓

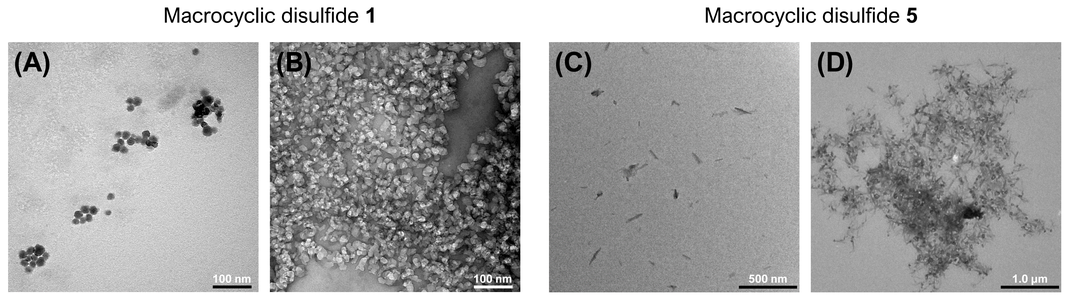

Figure 2. TEM images of peptides below and above CMC of peptides, respectively: (A & B) parent peptide **1**; and (C & D) Cit analogue **5**.

(A)

Name	Peptide Sequence	IC_{50} (μM)		
		E. coli	*B. subtilis*	*S. epidermidis*
1	*c*-[H-CIVIRFKFRC-NH_2]	38.2	34.2	19.4
[V^2]-**1** (**2**)	*c*-[H-C**V**VIRFKFRC-NH_2]	29.4	34.9	15.2
[V^4]-**1** (**3**)	*c*-[H-CIV**V**RFKFRC-NH_2]	37.5	36.4	19.1
[Cit^5]-**1** (**4**)	*c*-[H-CIVI**X**FKFRC-NH_2]	73.6	89.2	48.8
[Cit^9]-**1** (**5**)	*c*-[HCIVIRFKF**X**C-NH_2]	43.4	70.5	29.6

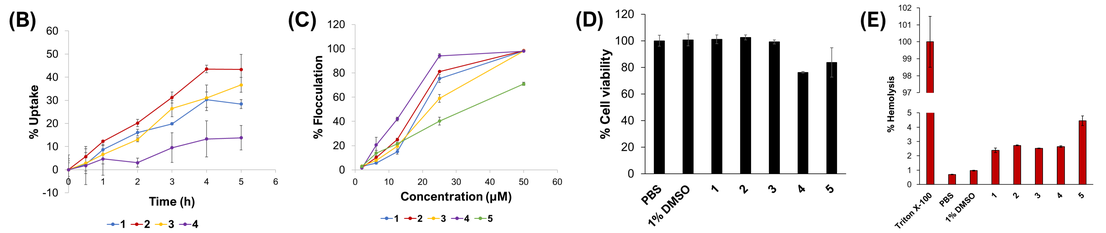

Figure 3. (A) **Inhibitory concentrations (IC_{50})** of peptides against different strains of bacteria (B) **Bacterial uptake** in *E. coli* of fluorescent-labelled peptides after 5-hour incubation. (C) **Concentration-dependent flocculation capacity** of *E. coli* suspensions after treatment and 4-hour incubation with peptides. PBS-treated samples were used as untreated controls. (D) **Cell viability of RAW 264.7 macrophages** after 24-hour incubation with 100 μM of each peptide. (E) **Percent hemolysis** in RBCs upon treatment with 100 μM of each peptide for 4 h. DMSO (1%) was used as a control in both studies. Error bars indicate the standard deviation of duplicates.

Conclusion & Future Work

In this study, **Cit-substituted analogues** of an antimicrobial β-sheet-forming macrocyclic peptide were designed and synthesized. Intrinsic fluorescence of Cit peptide **5** correlated with the ability to form large nanostructures due to greater conformational flexibility and more diverse secondary structures within the macrocycle. A network of urea hydrogen bonds between Cit residues may also be responsible for this effect.

- Insertion of Cit into macrocyclic disulfide **1** offers a novel means to enhance intrinsic fluorescence for future applications in biolabeling, biosensors, and drug delivery.
- Cit analogue **5** displayed a significant reduction in antimicrobial activity, compared to parent peptide **1**, which may be improved with further modifications to the peptide sequence.

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