DOI:10.18686/ahe.v8i8.13761

# Synthesis and Biological Evaluation of PEGylated Antimicrobial Peptide

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**Abstract:** Antimicrobial peptides (AMPs) possess strong antibacterial activity but are often associated with undesirable hemolytic activity, cytotoxicity, and sensitivity to proteolysis. Here, we report a simple and efficient prodrug system that combines antimicrobial peptides with photo-releasable PEG, successfully applied to the linear peptide indolicidin. After modification, this peptide significantly reduced hemolytic and cytotoxic effects while enhancing stability against proteolysis. Under irradiation conditions, the AMP is released, demonstrating antibacterial activity comparable to that of natural products.
**Keywords:** AMPs; Photo-releasable; PEGylation; Spatiotemporal release

**Fund Project:**
National Natural Science Foundation of China (No. 22307049)

## Introduction

Antimicrobial peptides (AMPs) are cationic and amphiphilic molecules found in various forms of life[1-2],exhibiting broad antibacterial activity effective against both Gram-positive and Gram-negative bacteria, viruses, and fungi[3].They reduce the risk of microbial resistance by disrupting the lipid bilayer of cell membranes, making them highly promising for the treatment of microbial infections[4], especially in the context of increasing bacterial resistance and diminishing efficacy of existing antibiotics[5]. However, the clinical application of natural antimicrobial peptides is often limited due to their strong hemolytic activity and cytotoxicity[6]. To reduce toxicity while maintaining antibacterial efficacy[7], researchers have proposed various strategies, including sequence modification, structural stabilization[8], and the development of antimicrobial peptide mimetics. In recent years, the emergence of light-regulated antibiotics has opened new avenues for antibiotic application[9-10], with "smart" antibiotics created using photo-switchable groups such as azobenzene and diene, which can be activated under specific light conditions to reduce side effects and the risk of resistance[11]. Additionally, the design of light-switchable antimicrobial peptides has also achieved success in in vivo studies. One approach to overcoming the limitations of peptide drugs is through covalent linkage with polyethylene glycol (PEG)[12-13]. PEGylated peptides can reduce renal clearance and immunogenicity, but they often significantly decrease biological activity. Releaseable PEGylation (rPEGylation) is a novel concept that combines peptides with PEG using cleavable linkers[14]. This study presents a prodrug system that effectively suppresses toxic side effects and enhances enzymatic degradation by linking AMPs with bulky mPEG through photounstable linkers, restoring antimicrobial activity after ultraviolet irradiation.

## 1. Instruments and Reagents

Fmoc-Pro-(2-CTC)-resin (1% crosslinked, 100-200 mesh, 0.476 mmol/g) and Rink Amide resin (1% crosslinked, 100-200 mesh, 0.91 mmol/g) were sourced from GL Biochem (Shanghai). All solvents and reagents were used as received. Analytical RP-HPLC was conducted on a Welch XB-C18 column (4.6 x 250 mm, 5 μm) with a linear gradient of $CH_3CN$ and $H_2O$ (0.1% TFA, v/v) at a flow rate of 1 mL/min for 30 minutes, detecting at 221 nm. For semi-preparative purification, a YMC-Pack ODS-A column (10 x 250 mm, 5 μm) was used with a specific $CH_3CN$ and $H_2O$ (0.1% TFA, v/v) gradient, at a flow rate of 2 mL/min.

# 2. Experimental Section

## 2.1 Peptide Synthesis

Preparation of AMP-mPEG Conjugates: We incorporated photo-labile amino acids during Fmoc solid-phase peptide synthesis, followed by adding side-chain photo-labile amino acids to the peptide. The final conjugate was formed through a terminal alkyne-azide "click" reaction between mPEG-azide and the photo-labile peptide. A photo-labile amino acid was synthesized using propargyl-functionalized 4,5-dimethoxy-α-methyl-nitro (DMNB) as the cage group, with the indole derivative assembled on Rink Amide resin via sequential HBTU-mediated coupling, using DiPEA as the base and DMF as the solvent. Fmoc deprotection was done with 20% piperidine. Resin cleavage was performed under 1% TFA/DCM to protect side-chain groups. The crude peptide underwent global deprotection with 90% TFA/H2O, precipitated in cold ether, and further purified by RP-HPLC. Finally, mPEG2000 was attached via "click" chemistry, yielding cIN-mPEG2000.

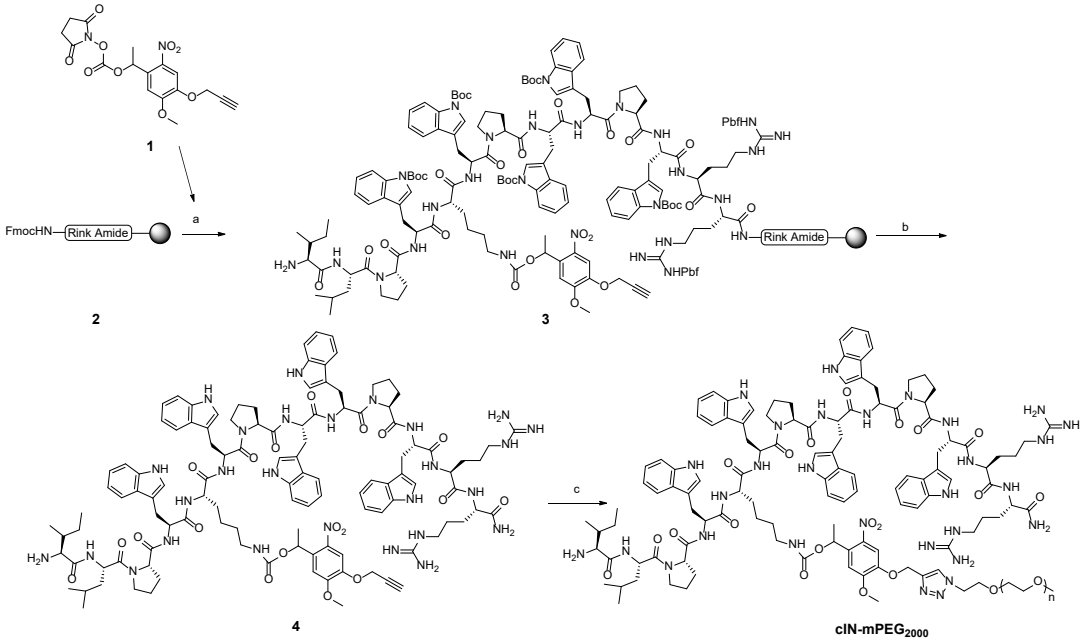



Figure 1. Synthesis of cIN-mPEG2000. Synthetic conditions: (a) Sequential coupling (Xaa, HBTU, DiPEA, DMF) and deprotection(20% piperidine/DMF, v/v) steps; (b) 95% TFA/H2O, v/v, 2 x 30 min, 8.7% yield for compound 15; (c) Sodium ascorbate,CuSO4.5H2O,, t-BuOH, H2O, 28%.

## 2.2 Stability Experiments:

Chemical Stability Experiment: cIN-mPEG2000 (0.1 mM) was added to acidic (pH 0.4) and basic (pH 13) buffer solutions and incubated at 37°C to simulate in vivo conditions. HPLC analysis was performed after incubation to assess degradation. Protease Stability Experiment: cIN-mPEG2000 was mixed with fresh or frozen mouse plasma and incubated at 37°C. A protease inhibitor was added at sampling to halt proteolysis, and HPLC was used to evaluate the stability of cIN-mPEG2000.

## 2.3 Cytotoxicity Experiment:

L02 cells ($1\times10^4$/well) were seeded in a 96-well plate and cultured for 24 hours. After washing with PBS, cells were treated with complete medium (10% FBS), starvation medium (1% FBS), and various treatments (AMP, AMP-mPEG2000, control) and incubated for 48 hours at 37°C with 5% $CO_2$. Thirty minutes before the end, 10 μL of CCK-8 reagent was added to each well, followed by 30 minutes of incubation. Cell proliferation and survival were assessed by measuring OD at 450 nm.

## 2.4 Antibacterial Experiment:

Escherichia coli (ATCC 25922), Staphylococcus aureus (ATCC 29213), and Enterococcus faecalis (ATCC 35667) were cultured in TBS at 200 rpm to the logarithmic phase. In a 96-well plate, 10 μL of GS or cGS-mPEG in DMSO was added to 90 μL of bacterial culture. DMSO (10%) served as a negative control. After a 48-hour incubation at 37°C, the minimum inhibitory concentration (MIC) was determined by measuring turbidity at 620 nm.

# 3. Results and Discussion

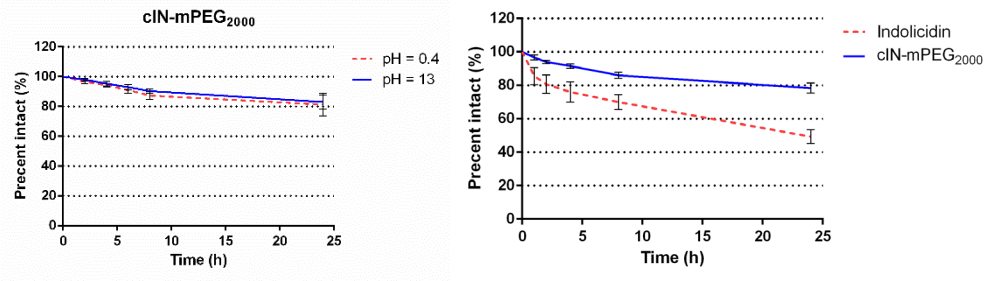
## 3.1 Synthesis Experiments:

Indolicidin: >95% purity, 18.7 mg, 9.8% overall yield from SPPS. MS (ESI+): m/z calcd for 1 /2 x C100H134N26O13 2+ : 953.53 1 /2 [M+2H] 2+ ; found: 954.15.

cIN-mPEG2000: >95% purity, 9 mg, 2.4% overall yield from SPPS. MS (ESI+): m/z calcd for 1 /4 x C202H322N30Na4O63 4+ : 1067.06 1 /4 [M+4Na] 4+ ; found: 1067.34.

### 3.2 Stability Experiments:

Chemical Stability Experiment: cIN-mPEG2000 remained stable after 24 hours under acidic (pH 0.4) and basic (pH 13) conditions, suggesting its potential in biomedical applications (Figure 2A). Protease Stability Experiment: cIN-mPEG2000 was incubated in mouse plasma. After 24 hours, about 50% of indolicidin degraded, while the stability of cIN-mPEG2000 was significantly enhanced with mPEG2000 (Figure 2B).

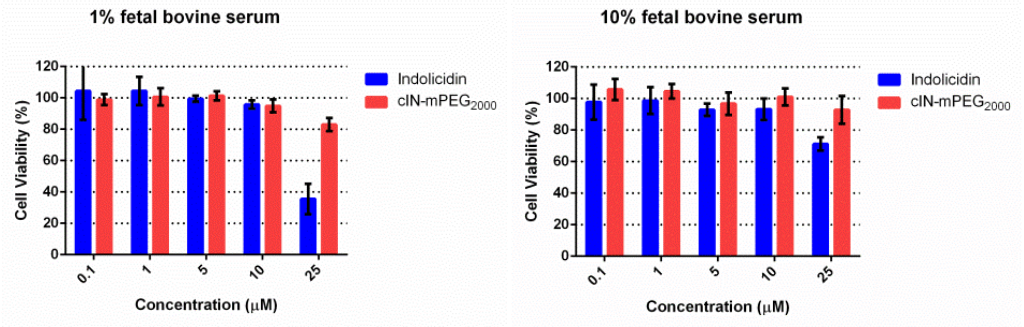



A B

Figure 2.(A)Chemical stability of cIN-mPEG2000 in 3% TFA (m/v) or 0.1 mM NaOH aqueous buffer . (B)Mouse serum stability of cIN-mPEG2000 in comparison to natural AMPs after 24 h incubation.

### 3.3 Cytotoxicity Experiment:

The cytotoxicity of the AMP-mPEG2000 conjugate was assessed in vitro against L02 cells. In the presence of 10% fetal bovine serum, no significant difference in cytotoxicity was observed between cIN-mPEG2000 and indolicidin (Figure 3A). However, in starvation medium, the toxicity of cIN-mPEG2000 was significantly lower than that of the natural peptide (Figure 3B).




A B

Figure 3. cIN-mPEG$_{2000}$ in comparison to natural AMPs under different concentrations. Cytotoxicity of AMPs, AMP-mPEG$_{2000}$ conjugates against L02 cells under both (B) 10% and C) 1% fetal bovine serum conditions.

### 3.4 Antibacterial Experiment:

The AMP's antibacterial activity was tested in both "caged" and "uncaged" forms against Gram-positive and Gram-negative bacteria (Table 1). The AMP-mPEG2000 conjugate showed reduced efficacy, but after irradiation, the "uncaged" AMPs regained activity comparable to natural products.

Table 1. Antimicrobial activity of peptides and AMP-mPEG2000 conjugates[a]

| Compound[a] | E.coli | S. aureus | E. faecium |
|---|---|---|---|
| Indolicidin | 25 | 12.5 | 12.5 |
| cIN-mPEG$_{2000}$ | 100[b] | 100[b] | 100[b] |
| Indolicidin + (NB-mPEG$_{2000}$)[c] | 25 | 12.5 | 12.5 |

[a] Measured using the minimum inhibitory concentration (MIC, μM);

[b] Maximum test concentration was 100 μM;

[c] After complete photolysis, the peptide was mixed in equimolar amounts with NB-mPEG2000 for antibacterial testing.

## 4. Conclusion

In summary, we have proposed a novel and efficient prodrug system that combines antimicrobial peptides (AMPs) with photo-

releasable PEG linkers for the first time. As a proof of concept, we developed the AMP prodrug cIN-mPEG2000, which restores antibacterial activity after irradiation with minimal side effects. Additionally, this AMP-mPEG2000 prodrug demonstrated high stability during serum incubation, indicating its potential for further in vivo applications. While we have only demonstrated the biological activity of light-triggered PEGylated AMPs, our work provides a new approach for designing various potential "smart" AMP conjugates targeting local microbial infections. However, the relatively shallow tissue penetration of UV irradiation remains a limitation for future in vivo applications. In the next steps, our research team will focus on employing two-photon absorption techniques to modify the light wavelength.

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