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Light-triggered Release of Antimicrobial Peptides from PEGylated Prodrugs

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Abstract: By synthesizing a light-sensitive AMP-mPEG prodrug with controllable release, we aimed to enhance drug bio-stability and antimicrobial efficacy. Incorporating light-shielding amino acids and employing a "click" reaction enabled mPEG conjugation to the AMP peptide chain. This study successfully designed and synthesized an AMP-mPEG prodrug, revealing restored antimicrobial activity post-irradiation. Findings provide insights into reversible PEGylation for antimicrobial peptides, serving as a reference for related research in international journals.

Keywords: Antimicrobial peptide; PEGylation; Prodrug; Hemolysis

Introduction

Antimicrobial peptides (AMPs), or host defense peptides, represent a class of cationic and amphipathic molecules present in various life forms, from microorganisms to humans.[1-3] The emergence of light-regulated antibiotics has provided a novel avenue for developing clinically applicable antimicrobials.[4] Antibiotics equipped with photo-switchable moieties like azobenzene and diarylethene can be locally activated under specified light radiation, thereby minimizing adverse effects and reducing the chances of microbial resistance development.[5-7]Another strategy to overcome limitations associated with peptide drugs involves covalent conjugation with polyethylene glycol (PEG). PEGylated peptides are known for advantages such as lower renal clearance, resistance to enzymatic degradation, and reduced immunogenicity; however, this often comes with a trade-off of diminished biological activity.[8-11]The concept of releasable PEGylation offers a potential solution.[12] This study demonstrates a prodrug system where AMPs, connected by a photosensitive linker mPEG, effectively suppress hemolytic side effects. Photoswitchable AMPs also have been designed and successfully applied to studies in vivo.

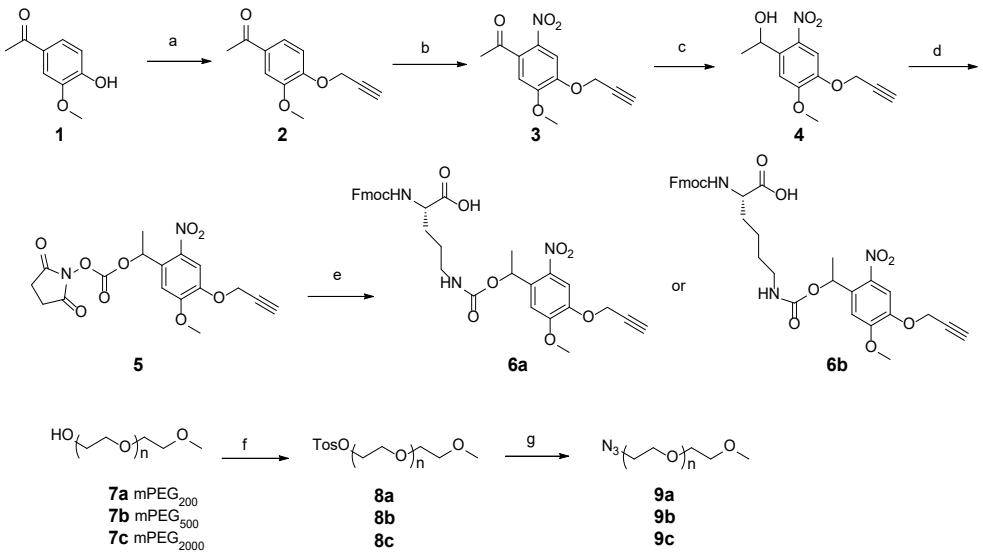
1. Instruments and Reagents

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 procured from GL Biochem (Shanghai) Co., Ltd. All other reagents and solvents were used without further purification. Analytical RP-HPLC was carried out on a Welch XB-C18 column (4.6 x 250 mm, 5μm) with a linear gradient of CH_3CN entering H_2O (0.1% TFA, v/v) at a flow rate of 1 mL/min over 30 minutes, with UV detection at 221nm. Semi-preparative RP-HPLC purification was performed on a YMC-Pack ODS-A column (10 x 250 mm, 5μm) using a gradient of CH_3CN entering H_2O (0.1% TFA, v/v) specific to each peptide at a flow rate of 2 mL/min.

2. Experimental Section

2.1 Synthesis Experiments

To prepare the AMP-mPEG conjugate, we employed a "click" reaction to link mPEG to the peptide chain,with mPEG-diazide reacting with the light-shielding peptide.



Scheme 1: Synthesis of Light-Shielding Amino Acids and mPEG Diazide

Synthesis conditions:(a) Propargyl-Br, K_2CO_3, ethanol, Δ, 91%;(b) KNO_3, TFA, 78%;(c) $NaBH_4$, methanol, 1,4-dioxane, 96%;(d) DSC, triethylamine, acetonitrile, 96%;(e) Fmoc-Orn-NH_2 for 6a and Fmoc-Lys-NH_2 for 6b, DiPEA, acetonitrile, dichloromethane, with yields of 84% and 75%, respectively;(f) TsCl, triethylamine, toluene, dichloromethane, with yields of 93%, 68%, and 54% for 8a, 8b, and 8c, respectively;(g) NaN_3, DMF, Δ, with yields of 89%, 78%, and 71% for 9a, 9b, and 9c, respectively.

Scheme 2: Sodium ascorbate, $CuSO_4$.5H2O, 9, t-BuOH, H_2O, 43%, 36% and 25% yield for cGS-mPEG$_{200}$, cGS-mPEG$_{500}$ and cGS-mPEG$_{2000}$ respectively.

2.2 Hemolysis Assay:

Sheep blood, 10% (w/v) SDS, and PBS solutions were freshly prepared. AMP and AMP-mPEG conjugates were dissolved in DMSO at 4 mM concentration. These solutions were then half-diluted in DMSO . Fresh sheep blood was diluted to 5 x 10^5 red blood cells per milliliter. Each well received 5µL of peptide, 45µL of PBS, and 50 µL of diluted blood. DMSO served as a negative control, while SDS (0.1% w/v) acted as a 100% hemolysis control. The experiment was triplicated, incubated at 37 degrees Celsius for 30 minutes, and red blood cells were separated by centrifugation (300 rpm, 10 min). Each supernatant (50 µL) was transferred to a new 96-well microplate for turbidity measurement at 450 nm.

2.3 Antimicrobial Assay:

Experiments were conducted using Escherichia coli (ATCC 25922), Staphylococcus aureus (ATCC 29213), and Enterococcus faecalis (ATCC 35667). Bacteria were cultured in TBS medium at 200 rpm until reaching mid-logarithmic growth phase. In a 96-well microplate, 10 µL of GS or cGS-mPEG solution in DMSO and 90 µL of mid-logarithmic phase bacterial culture were added. And DMSO (10% v/v) was used as a negative control. After 48 hours of incubation at 37 degrees Celsius, the minimum inhibitory concentration (MIC) was assessed by measuring turbidity at 620 nm.

3. Results and Discussion

3.1 Synthesis Experiments:

cGS-mPEG$_{200}$: >95% purity, 8 mg, 11.5% overall yield from SPPS. MS (ESI+): m/z calcd for $C_{82}H_{123}N_{16}O_{20}^+$: 1651.91 [M+H]+; found: 1650.31.

cGS-mPEG$_{500}$: >95% purity, 6 mg, 7.3% overall yield from SPPS. MS (ESI+): m/z calcd for $C_{94}H_{146}N_{16}O_{26}K^+$: 1954.02 [M+K]+; found: 1954.09.

cGS-mPEG$_{2000}$: >95% purity, 10 mg, 7.0% overall yield from SPPS. MS (ESI+): m/z calcd for 1/2 x $C_{164}H_{286}N_{16}Na_2O_{61}^{2+}$: 1750.98 1/2 [M+2Na]2+; found: 1751.26.

3.2 Hemolysis Assay:

We observed that mPEG$_{200}$ and mPEG$_{500}$ failed to sufficiently inhibit hemolytic activity except for a slight decrease observed with cGS-mPEG$_{500}$ at 50 µM. Encouragingly, cGS-mPEG$_{2000}$ exhibited negligible hemolytic effects at concentrations below 200 µM, suggesting its potential candidacy as a prodrug for further investigation (Figure 1).

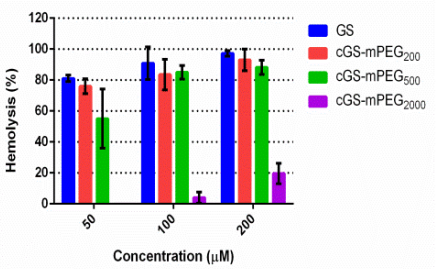


Figure 1. Hemolytic activity of cGS-mPEG conjugate compared to native AMP at different concentrations.

3.3 Antimicrobial Assay:

Using three bacterial strains, including both Gram-positive and Gram-negative, the antimicrobial capabilities of AMPs in "caged" and "uncaged" forms were measured (Table 1). As expected, both AMP-mPEG$_{2000}$ conjugates exhibited abolished antimicrobial activity, while the "uncaged" AMPs regained antimicrobial activity post-irradiation, comparable to the natural products. The testing included the byproduct NB-mPEG$_{2000}$ to exclude its impact on antimicrobial assays. With increased irradiation intensity, the release kinetics would significantly enhance, substantially reducing drug release duration.

Table 1. Antimicrobial Activity of Peptides and AMP-mPEG$_{2000}$ Conjugates[a]

Compound[a]	*E.coli*	*S. aureus*	E. faecium
GS	25	3.13	3.13
cGS-mPEG2000	100[b]	100[b]	100[b]
GS + (NB-mPEG2000)[c]	25	3.13.	6.25

[a] Measured in minimum inhibitory concentration (MIC, μM);[b] The maximum test concentration was 100 μM;[c] Post complete photolysis, equimolar mixtures of peptides and NB-mPEG$_{2000}$ were used for antimicrobial assays.

4. Conclusion:

In summary, we have introduced a concise and efficient prodrug system that links antimicrobial peptides (AMPs) with photo-releasable polyethylene glycol (PEG). We developed two AMP prodrugs, cGS-mPEG$_{2000}$, which successfully restored antimicrobial activity with minimal side effects post-irradiation. Both AMP-mPEG$_{2000}$ prodrugs exhibited high stability in serum, and the released byproducts were non-toxic to human cells, demonstrating potential for further in vivo applications. While we presented examples of photo-triggered bioactivity for two PEGylated AMPs, our work opens avenues for designing various "smart" AMP conjugates to combat local microbial infections. Although in vivo application is currently limited by the penetration depth of UV radiation, we aim to explore this aspect in our future work.

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