

DEVELOPMENT OF LIPIDATED ANTIFUNGAL PROTEINS AND IONIC LIQUID-BASED MICROEMULSIONS FOR TOPICAL TREATMENT OF SUBCUTANEOUS FUNGAL INFECTIONS

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(皮下真菌感染症の局所治療のための脂質修飾抗真菌タンパク
質およびイオン液体を構成要素とするマイクロエマルジョ
ンの開発)

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論 文 内 容 の 要 旨

The rising incidence of fungal infections, particularly pathogens invade the barrier of the skin, and the emergence of drug-resistant fungal strains have become pressing global health concerns. Morbidity and mortality associated with fungal diseases continue to increase annually, largely due to the limited efficacy of current antifungal agents and their cytotoxicity toward human cells. Chitinase exerted antifungal properties by degrading the chitin layer in the fungal cell wall. However, the effectiveness of chitinase is limited by the requirement for high concentrations to achieve antifungal activity. Therefore, there is a critical need to develop safer antifungal therapies.

In this dissertation, I present the development of lipidated antifungal protein and ionic liquid-based microemulsions for topical treatment of subcutaneous fungal infections. Chapter 1 explains about the general introduction. In Chapter 2 describes the role of lipid modifications in regulating chitinase domain function, enabling to improve the antifungal activity, I concerned on the basic role of lipid moiety covalently conjugated to chitinase domain in enhancing the antifungal efficacy of chitinase domains. LysM and CatD with a peptide containing glutamine residue (Q-tag), which were conjugated with lipid-fused peptides containing lysine residue (K-tag) using microbial transglutaminase (MTG) conjugation, were explored to understand how different lipid moieties affect antifungal activity. The efficacy of the lipidated chitinases varied significantly depending on the attached lipid type. Palmitoylation (LysM-Pal and CatD-Pal) resulted in stronger antifungal effects against *Trichoderma viride* than cholesterylation (LysM-Chol and CatD-Chol). This enhanced activity is likely due to the lipid moiety's ability to efficiently anchor into the fungal plasma membrane, thereby facilitating membrane destabilization. In contrast, cholesterylated chitinases exhibited lower enhancement of the antifungal activity than palmitoylated chitinase, potentially due to poor membrane interaction caused by aggregation of the cholesteryl groups. Nevertheless, cholesterylation of chitinase

domains remains a promising strategy for targeting fungal hyphae. This approach may enhance delivery of LysM or CatD to chitin-rich regions, thereby promoting degradation or disruption of the fungal cell wall.

In chapter 3, I focused on employing chitin binding domain modified with lipid for topical application by incorporating the artificial lipidated antifungal LysM-based proteinaceous drugs into a choline-based, biocompatible ionic liquid-in-oil microemulsion formulation (IL/O MEF), resulting in a LysM-lipid-loaded MEF. Among the various formulations evaluated (LysM-C12- and LysM-C16-loaded MEF), the LysM-C14-loaded MEF demonstrated superior skin permeability and the most potent antifungal activity in an *in vitro* topical antifungal assay against *T. viride* growing actively beneath the skin. MTG-mediated conjugation of a C14 alkyl chain to LysM, in combination with an IL/O MEF, significantly enhanced the transdermal delivery of LysM-C14 across mouse skin. Stability assessments revealed that lipidated LysM, along with its dispersion in the MEF, effectively prevented protein aggregation within the IL/O system, thereby preserving antifungal activity. Collectively, our findings suggest that surface-active ionic liquid (SAIL)-based MEFs represent promising carriers for the topical delivery of protein-based antifungal therapeutics, as evidenced by their robust performance in a mouse model of subcutaneous skin infection.

In chapter 4, I further explored another potential antifungal polypeptide-based formulation using a recently developed ILO/MEF for topical treatment of subcutaneous fungal infections. The formulation, incorporated into an IL/O MEF, was evaluated *in vitro* for its ability to enhance skin penetration and its antifungal efficacy against *T. viride*. Our results demonstrated that the IL/O MEF markedly improved the topical permeation of ϵ -poly-L-lysine (EPL), and completely inhibited the growth of *T. viride* beneath the skin. This enhanced delivery is likely attributed to a combination of strong electrostatic interactions between the polycationic EPL and the choline propionate ([Cho][Pro]) ionic liquid, and the inherent membrane-disruptive properties of the IL components, which may persist as a complex even after penetrating the stratum corneum. While EPL served as the model antifungal peptide in this study, the platform is potentially applicable to other polycationic and amphiphilic peptides, such as poly-L-ornithine and poly-L-arginine, as well as a broader range of biopharmaceutical agents. In summary, our biocompatible SAIL-based IL/O MEF provides a promising strategy for the effective topical delivery of peptide-based antifungal therapeutics.

In conclusion, these findings demonstrated encapsulation strategies into IL/O MEF that enhanced antifungal activity of protein-lipid conjugate and polycationic peptide against *T. viride* beneath the skin was successfully developed. Lipidation of protein possess potentials to be used not only to increase the hydrophobicity and the binding affinity of protein to the cell membrane, leading to disruption of the membrane stability, resulting in fungal cell lysis, but also to enhance effectively the skin permeation of protein-lipid conjugate. This system holds excellent potential in the development of therapeutic biopharmaceuticals.