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Localization of antimicrobial peptides on polymerized liposomes leading to their enhanced efficacy against *Pseudomonas aeruginosa*[†]

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Abstract

Antimicrobial peptide IG-25 (a truncated version of LL-37 of the cathelicidin family) tethering an azido-capped poly(ethylene glycol) chain at the N-terminus was site-specifically attached to alkynyl-terminated polymerized liposomes using copper catalyzed “click” reaction, leading to an 18 fold enhancement in efficacy against *Pseudomonas aeruginosa* when compared to LL-37 without any increase in cytotoxicity to human corneal epithelial cells.

Cationic antimicrobial peptides (AMPs) have recently attracted tremendous research interest.^{1,2} AMPs are found in virtually all living organisms. They are a first line of defense against invading pathogens. The low susceptibility for pathogens to develop resistance to AMPs has generated immense interest in their potential use as new therapeutics against multidrug resistant pathogens. Unfortunately, *in vitro* studies show that AMPs require a high concentration (in the micromolar range) to exhibit effective antimicrobial activity. Slightly above this threshold concentration, they often become toxic to host cells.³ In this work, we demonstrate that localization of a modified AMP (N₃-EG₁₂-IG-25, Fig. 1) onto polymerized liposomes (PL) by site-specific covalent attachment can greatly enhance the antibacterial activity of the AMP without increasing the cytotoxicity to host cells.

Several mechanistic models suggested that following the initial binding of the polycationic AMPs to the negatively charged bacterial membrane,⁴ the subsequent disruption of the membrane requires a cooperative action of many AMP molecules in high local concentration at the membrane.⁴ A relatively high concentration of isolated AMPs in the solution is then needed to accelerate this process for rapid killing of bacteria that may replicate themselves every 20 min. Alternatively, a high local concentration of AMPs at the bacterial surface may be achieved by pre-localization of AMPs on a scaffold before binding to bacteria. Also, localization of AMPs should greatly enhance the binding to the bacteria through collective electrostatic interactions. Indeed, immobilization of AMPs on solid substrates⁵ has led to enhanced antimicrobial activity in some systems, such as polymers⁶ and titanium.⁷ While substantial studies relevant to anti-infection on medical devices have been performed on solid substrates, localization of AMPs on water soluble scaffolds for potential application as new antibiotic drugs has received far less attention. Limited examples include AMP-modified silver nanoparticles,⁸ quantum dots,⁹ soluble polymers,¹⁰ micelle nanoparticles formed by self-assembly of AMP-modified block copolymers¹¹ and

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cholesterol.¹² While the reported synthesis of water-soluble AMP-polymers was cumbersome and time-consuming,¹⁰ the AMP-tagged micelles were readily prepared, but the high density of charged AMPs on the non-polymerized micelles may limit their stability. A moderate enhancement in the antimicrobial efficacy by two¹¹ to six¹² fold was reported on these systems. On the other hand, the use of silver nanoparticles and quantum dots as a scaffold has an issue of cytotoxicity on their own.¹³ In this work, we use polymerized liposome¹⁴ (PL) as a scaffold for covalent localization of AMPs. This scaffold is nontoxic, highly stable against disruption, easily prepared, and allows precise control on particle size.¹⁴ The results show a 18 fold increase in efficacy of IG-25 when compared to LL-37 without any increase in cytotoxicity.

The polymerized liposome (Fig. 1) is readily prepared from 10,12-pentacosadiynoic acid (**1**, ESI†). Briefly, an aqueous solution of the lipids **1** was extruded 20 times through a 100 nm filter. The resultant liposomes were polymerized under 254 nm UV at 0 °C for 1 hour. After purification with a size exclusion column (PD SpinTrap™ G-25 from GE Healthcare), the carboxylic acid terminated polymerized liposome (PL-COOH) with an average diameter of ~110 nm was obtained.

We chose IG-25¹⁵ (Fig. 1) to attach to PL. IG-25 is a truncated form of LL-37, an extensively studied human AMP of the cathelicidin family.¹⁶ Gabriel *et al.* have shown that the conjugation method can directly affect the antibacterial activities of AMPs immobilized on titanium surfaces.⁷ Attaching the peptide specifically at its terminus *via* a long and flexible PEG linker provided the highest bactericidal efficacy.⁷ Therefore, we used a dodeca(ethylene glycol) linker for tethering IG-25 to PL. The linker was incorporated at the N-termini of IG-25 at the end of the solid state synthesis of the peptide. The other end of the EG₁₂ linker was capped with an azido group for site-specific attachment to an alkynyl-terminated scaffold *via* copper-catalyzed azide-alkyne cyclization (CuAAC, a “click” reaction).¹⁷ For a comparison, we also attached the peptide to carboxylic acid-terminated PL¹⁸ (PL-COOH) *via* electrostatic interactions (Fig. 1).

To tether IG-25 on PL *via* the “click” reaction, PL-COOH was first converted to an alkyne-terminated (“clickable”) PL (CPL) by attaching water-soluble propargyl amine using carbodiimide chemistry (Fig. 1). The reaction was carried out in acetate buffer using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and *N*-hydroxysulfosuccinimide (sulfo-NHS) as the coupling reagents. The resultant CPL was purified by size exclusion chromatography (PD SpinTrap™ G-25 from GE Healthcare).

The “click” immobilization of N₃-EG₁₂-IG-25 onto CPL was performed using CuSO₄ and sodium ascorbate (reducing agent) to generate Cu(I) catalyst *in situ*. The redox cycles of Cu(I)/Cu(II)/O₂/ascorbate produces reactive oxy species and electrophiles causing oxidative damage and aggregation of proteins and peptides.¹⁹ We have found that the click reaction performed under anaerobic conditions using appropriate Cu(I) ligand as co-catalyst not only greatly limits the side reactions but also enhances the rate of the click reaction. The co-catalyst that performed the best under anaerobic conditions was the water-soluble bis-triazole ligand **2** (Fig. 1); the results for which will be published elsewhere. Ligand **2** also serves as a sacrificial reductant by reducing the reactive oxy species such as hydrogen peroxide, thereby shielding the biological moieties from oxidative damage.¹⁹ The CuAAC reaction was performed with excess of N₃-EG₁₂-IG-25 (0.91 mM), ligand **2** as co-catalyst (0.65 mM), sodium ascorbate (6.3 mM) and CuSO₄ (0.33 mM) in PBS buffer in an anaerobic chamber for 1 h to give the peptide terminated PL (PL-EG₁₂-IG-25). The product

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was purified by passing it through size exclusion chromatography (PD SpinTrap™ G-25 from GE Healthcare) twice to remove excess reagents and copper species.

Fluorescamine was used for determining the concentration of N₃-EG₁₂-IG-25 bound to the PL by fluorometric assay of the primary amine.²⁰ Fluorescamine reacts quantitatively with primary amines to yield a highly fluorescent product. The fluorescence intensities of the resulting solution can be used to estimate the amount of primary amine present in the solution by a calibration curve which has been previously obtained by reacting a series of concentrations of glycine with fluorescamine (ESI†).

The yield for the CuAAC reaction on PL was determined to be 11%, based on the amount of 10,12-pentacosadiynoic acid used for the preparation of PL-COOH. The low yields are caused by the non-availability of the functional groups on the inner lipid layer of the liposome and the steric bulkiness of IG-25, as well as the electrostatic repulsion of the polycations on IG-25.

Attachment of N₃-EG₁₂-IG-25 to PL-COOH by electrostatic interaction to form PL/N₃-EG₁₂-IG-25 was straightforward. At physiological pH, PL-COOH carries a net negative charge on its surface, thus attracts the positively charged N₃-EG₁₂-IG-25 *via* electrostatic interaction. Specifically, an excess of IG-N₃-EG₁₂-IG-25 was added to a solution of PL-COOH in PBS buffer, and the solution was incubated at room temperature for 2 h in PBS. The sample was then purified by size exclusion chromatography (PD SpinTrap™ G-25 from GE Healthcare). Fluorescamine assay showed ~3% attachment of the peptide to PL-COOH. The low coverage may be due to the screening of negative charge on PL-COOH by the already attached, bulky, multiple charged N₃-EG₁₂-IG-25. Also, there may be some loss of peptide during purification.

The antimicrobial activity of the two AMP-PL conjugates was evaluated by measuring the half maximal effective concentration (EC₅₀) of IG-25 against *Pseudomonas aeruginosa* (strain PA01, see ESI† for details of the assay). PA is the most common cause of contact-lens related eye infection. The bulk concentrations of the IG-25 attached to the PL were determined by the above fluorescamine assay. In addition to the two AMP-PL conjugates, the EC₅₀ of the free N₃-EG₁₂-IG-25 and LL-37, as well as the liposome CPL scaffold, were also measured. The results are summarized in Table 1. Note that the CPL control was subjected to the identical click reaction conditions (except the absence of the AMP N₃-EG₁₂-IG-25) and to the same purification procedure. This control experiment established that the remaining trace amount of copper, if any, did not have detectable toxicity to PA.

Table 1 shows that the click attachment of the AMP N₃-EG₁₂-IG-25 specifically at the N-terminus *via* a PEG linker to PL greatly enhanced the EC₅₀-based potency by 54 fold with respect to free N₃-EG₁₂-IG-25. In comparison, the PL/N₃-EG₁₂-IG-25 construct prepared by electrostatic immobilization of N₃-EG₁₂-IG-25 on PL-COOH did substantially improve the potency by 6 fold, in comparison to N₃-EG₁₂-IG-25 but the enhancement was far less than by the click immobilization. The results suggest that covalent attachment of AMP on PL leads to higher activity than attachment achieved by electrostatic association. It also indicates that the long, flexible PEG linker and the site specific attachment of PL-EG₁₂-IG-25 may facilitate the cooperative action of the localized AMP for disruption of the bacterial membrane.

To assess the toxicity of PL-EG₁₂-IG-25 to host cells, cell viability assay was performed on a human corneal epithelial cell line (SV40-HCEC).²¹ The effect of PL-EG₁₂-IG-25 on cell growth was negligible as compared to the control (liposome without the presence of N₃-EG₁₂-IG-25) in the concentration range of 500 µg mL⁻¹. Moreover, cell viability was not

affected even at a concentration of 5.2 μ M (based on amount of IG-25 attached on the liposome).

In conclusion we have demonstrated that localization of the AMP IG-25 by specific attachment of its N-terminus *via* a PEG linker to PL significantly enhanced its EC₅₀-based potency by 54 fold in comparison to N₃-EG₁₂-IG-25. The IG-25 localized in this way was also 18 times more active against PA01 than its parent AMP, LL-37. Furthermore, preliminary results show that the cytotoxicity of the AMP to SV40-HCEC cells did not increase by the localization.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Representation of the modified AMP (N_3 -EG₁₂-IG-25, analogue of LL-37) and the illustration of its attachment through direct electrostatic binding to the polymerized liposome (PL-COOH), or through click reaction onto the “clickable” polymerized liposome (CPL).

Table 1Summary of the EC₅₀ values of the IG-25 derivatives and LL-37 against PA01

AMP	EC ₅₀ /μM
PL-EG ₁₂ -IG-25	0.421
PL/N ₃ -EG ₁₂ -IG-25	3.71
Free N ₃ -EG ₁₂ -IG-25	23.0
Free LL-37	7.67
CPL control	NA ^a

^aNo killing was detected.