Supplementary Material (ESI) for Molecular BioSystems

Supporting Information for

Localization of Antimicrobial Peptides on Polymerized Liposomes Leading to Their Enhanced Efficacy against *Pseudomonas aeruginosa*

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Reagents

Compound 10,12- pentacosadiynoic acid (**1**) was purchased from Alfa Aesar (Ward Hill, MA), Sulfo-NHS was purchased from Sigma Aldrich *(*Milwaukee*,* WI)**, IG-25** was custom-synthesized by Alpha Diagnostic International (San Antonio, TX). **LL-37** was purchased from the American Peptide Company (Sunnyvale, CA). Polymerized liposomes were synthesized using an extruder purchased from Avanti Polar Lipids, Inc. (Alabaster, Alabama). All deoxygenated solutions were obtained by bubbling nitrogen through them and stored inside an anaerobic chamber.

Synthesis of Carboxy-terminated Polymerized Liposomes (PL-COOH)

Carboxy-terminated lipid **1 (**30 mg) were dissolved in $CHCl_3$ (10 mL) in a 25 mL conical flask. The solvent was evaporated by rotary evaporator and dried under vacuum pump for 10 min. The transparent lipid film on the wall of the flask was dispersed in 10 mL of 0.01 M PBS buffer at room temperature. The resultant aqueous suspension was sonicated for 1 h at 60 ˚C. The sample was extruded 20 times at 60˚C using an Avanti mini extruder with a 100 nm Whatman Nuclepore Track-Etch Membrane filter. The vesicles were polymerized using UV lamp at 254 nm for 1 hour at 0 ˚C in a petri dish yielding a faint reddish brown solution and purified by size exclusion column twice (PD SpinTrap™ G-25 from GE Healthcare). The size of the particles in 0.01 M PBS buffer was measured by dynamic light scattering (Brookhaven 200 SM goniometer and correlator). The liposomes prepared from various batches were homogeneous in size and exhibited an average diameter of about 110 nm.

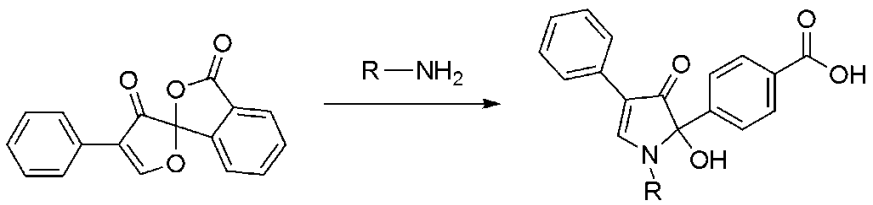
Synthesis of Sulfo-NHS-terminated Polymerized Liposomes (PL-COOSNHS)
A 1.0 mg sample of sulfo-NHS (Sigma-Aldrich, St. Louis, MO) and 1.5 mg of EDC **(**Alfa Aesar, Ward Hill, MA) were added to a 1.7 mL Eppendorf tube carrying 260 μL of 3 mg/mL sample of **PL-COOH** in 0.01 M PBS buffer. The resulting solution was mixed well and allowed to stand for 4 h under ambient conditions. Excess of the unreacted species was removed by size exclusion column twice (PD SpinTrap™ G-25 from GE Healthcare).

Synthesis of Alkyne-terminated Polymerized Liposomes (CPL)
To the purified solution of 130 μL of 3 mg/mL sample of sulfo-NHS terminated polymerized liposome in 0.01 M PBS buffer was added 1.0 mg of propargyl amine hydrogenchloride **(**Alfa Aesar, Ward Hill, MA). The solution was mixed well and allowed to stand for 6 h under ambient conditions. Excess of the unreacted species was removed by passing through a size exclusion column twice (PD SpinTrap™ G-25 from GE Healthcare).

Conjugation of N_3-EG_{12}-IG-25 to CPL *via* the CuAAC Reaction
To the deoxygenated solution of 130 μL of 3 mg/mL sample of **CPL** in 0.01 M PBS buffer was added 130 μL 10 mg/mL deoxygenated solution of azide-terminated **IG-25** (**N_3-EG_{12}-IG-25**) (custom synthesized by Alpha Diagnostic International Inc., San Antonio) in 0.01 M PBS buffer. To the resulting solution was added 130 μL of deoxygenated solution containing $CuSO_4$ (975 μM), ligand **2** (1.95 mM) and sodium ascorbate (18.75 mM) in 0.01 M PBS buffer. The solution was thoroughly mixed and left to stand for 1 h under anaerobic conditions. The reaction mixture was taken out of the anaerobic chamber and purified by passing through a size exclusion column twice (PD SpinTrap™ G-25 from GE Healthcare) to obtain **PL-EG_{12}-IG-25**. All the steps in the reaction were carried out inside an anaerobic chamber.

Conjugation of N_3-EG_{12}-IG-25 to PL-COOH *via* Electrostatic Interaction
To the purified solution of 130 μL of 3 mg/mL sample of **PL-COOH** in 0.01 M PBS buffer was added 130 μL 10 mg/mL solution of **N_3-EG_{12}-IG-25** in 0.01 M PBS buffer. The solution was thoroughly mixed and left to stand for 2 h under ambient conditions. The reaction mixture was purified by passing through a size exclusion column twice (PD SpinTrap™ G-25 from GE Healthcare) to obtain **PL/N_3-EG_{12}-IG-25**.

Procedure for Obtaining Calibration Curve for Calculation of the Yields for N_3-EG_{12}-IG-25 Conjugation to Polymerized Liposome



Glycine (2 mg) was dissolved in 1.5 mL of 0.01 PBS buffer and added to a 1.5 mL, DMSO solution of fluorescamine (10 mg/mL, purchased from Sigma-Aldrich). The solution was mixed thoroughly and left to stand for 15 minutes under ambient conditions to obtain the highly fluorescent product. The fluorescence intensity (λ_{ex} = 365 nm; λ_{em} = 460 nm) of the resulting solution was recorded at various dilutions by a fluorometer (Perkin-Elmer/Packard, Wellesley, MA). The plot of concentration of glycine *versus* fluorescence gave a calibration curve against which the amount of **IG-25** conjugated to the polymerized liposomes was calculated. Since **IG-25** contains 13 amino groups, the reading obtained from modified liposomes were divided by 13. Also, each purification with size exclusion column gave an approximate recovery of 90%, hence the yields were adjusted accordingly. Since all liposomes after conjugation were purified 7 times through the size exclusion columns, the yields were multiplied with factor of 2.09. Also, due to non-availability of half the functional groups trapped inside the inner sphere of doubled layer liposome, the yields were multiplied by a factor of two.

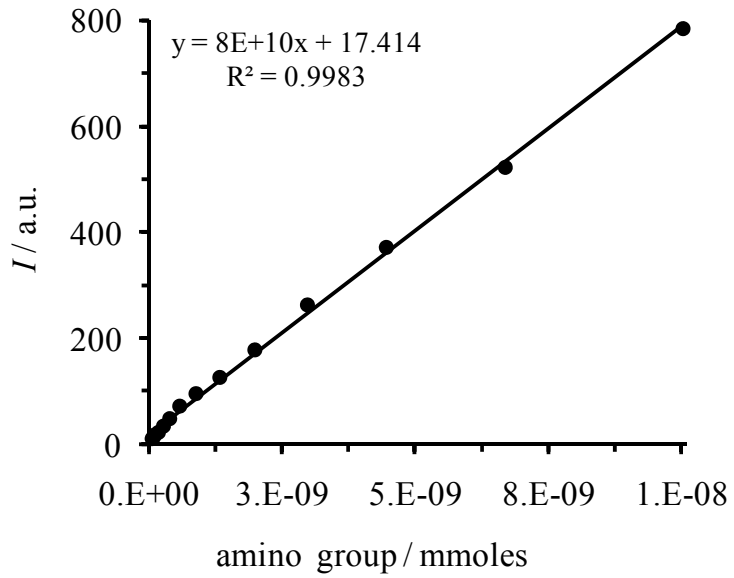


Figure S1. Fluorescence intensity (*I*) (λ_{ex} = 360 nm, λ_{em} = 465 nm) *versus* concentration of glycine in PBS (pH = 7.2), served as a calibration curve for quantification of the **N_3-EG_{12}-IG-25** attached on the polymerized liposomes after the reaction.

Table S1. Summary of the % Yields Obtained for Different Modes of Conjugation

AMP (method for conjugation to PL)	% Yields after adjustments μM
PL-EG_{12}-IG-25 (CuAAC)	11.2 ± 0.004
PL N_3-EG_{12}-IG-25 (Electrostatic)	1.0 ± 0.24

Procedure for Measuring the Antimicrobial Activity[35] of N_3-EG_{12}-IG-25 Conjugated to Polymerized Liposome (PL)

One single isolated PA01 colony was inoculated in 5 mL Luria-Bertani Broth (LB) overnight at 37°C in shaker at 250 rpm. A 1.0 mL of the bacterial suspension was used to inoculate 50 mL of fresh LB. The inoculum was then incubated with vigorous shaking (250 rpm) at 37°C for 2 h to achieve mid-log phase growth. After 2 h, 25 mL sample from the liquid culture was transferred to a 50 mL conical tube and centrifuged at 3100 rpm for 10 minutes. The resultant bacterial pellet formed after centrifugation was resuspended in 4 mL of phosphate buffer (PB; 8.2 mM Na_2HPO_4, 1.8 mM KH_2PO_4 (pH 7.4)). The optical density (OD) of the suspension was adjusted to 0.1 at 660 nm by adding an appropriate volume of Phosphate Buffer. The bacterial suspension (10 µL, approximately 10^8 cfu/mL) was mixed with 5 µL of liposome (the different concentrations of modified liposomes prepared prior to mixing include: 1, 0.5, 0.2, 0.1, 0.05 and 0.01 mg/mL) and 35 µL of PB buffer in a 1.7 mL Eppendorf tube. It was noted that the same concentration range was prepared for all the modified liposome obtained using the different conjugation methods. The resulting solution was incubated for 2 hours at 37°C with vigorous shaking. After incubation, serial dilution of each liposomal-bacterial solution was made. 10 µL of each dilution was used to inoculate agar plates. After inoculation the plates were incubated at 37°C overnight. After incubation the plates were removed and the number of colonies counted. The results obtained were graphed and are shown below.

Results Obtained *via* the Antimicrobial Peptide Assay

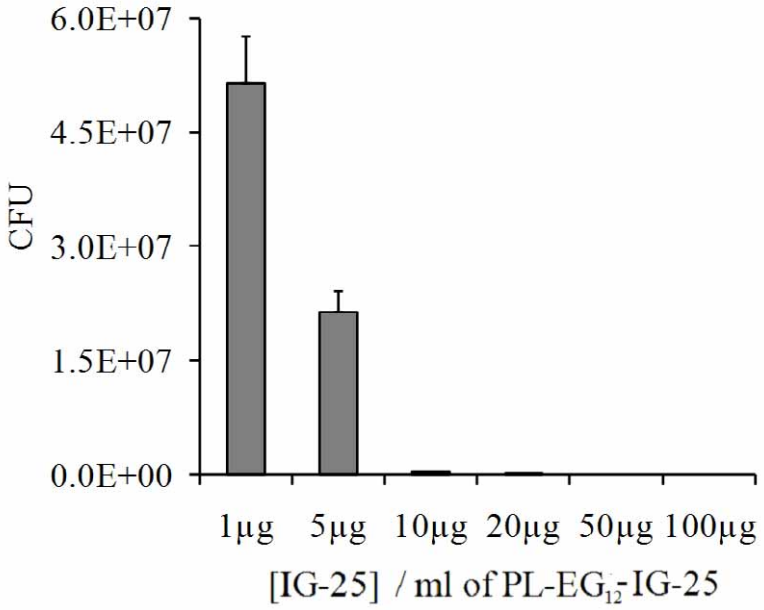


Figure S2. Bar graph represents colony forming units (CFU) obtained during antimicrobial peptide assay for various dilutions of **PL-EG$_{12}$-IG-25**. The x-axis shows the amount of **IG-25** per mL, carried on surface by **PL-EG$_{12}$-IG-25** in a given solution.

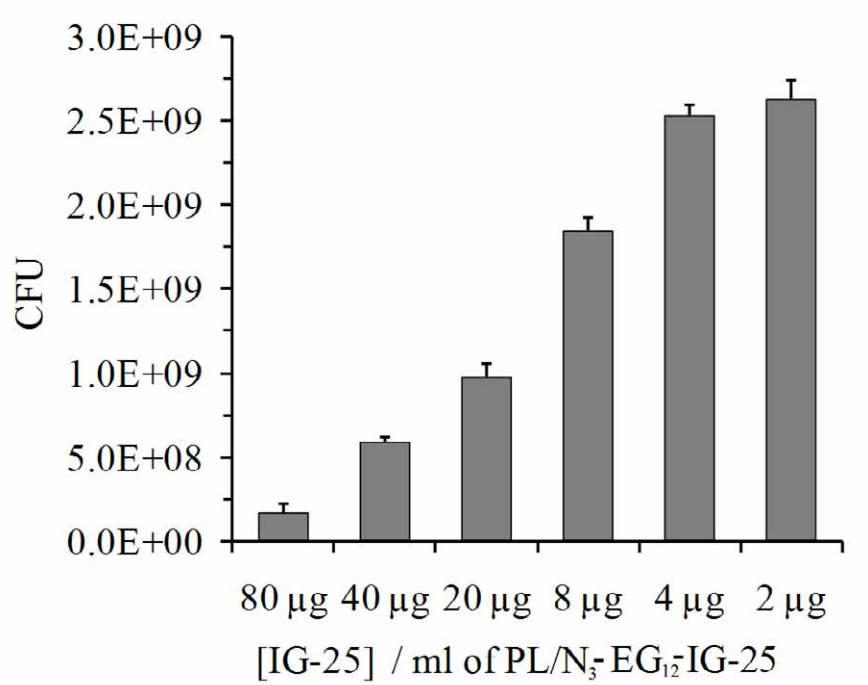


Figure S3. Bar graph represents colony forming units (CFU) obtained during antimicrobial peptide assay for various dilutions of **PL/N$_3$-EG$_{12}$-IG-25**. The x-axis shows the amount of **IG-25** per mL, carried on surface by the **PL/N$_3$-EG$_{12}$-IG-25** in a given solution.

EC_{50} Calculations

EC_{50} calculations were performed from the colony count data acquired using SBE software

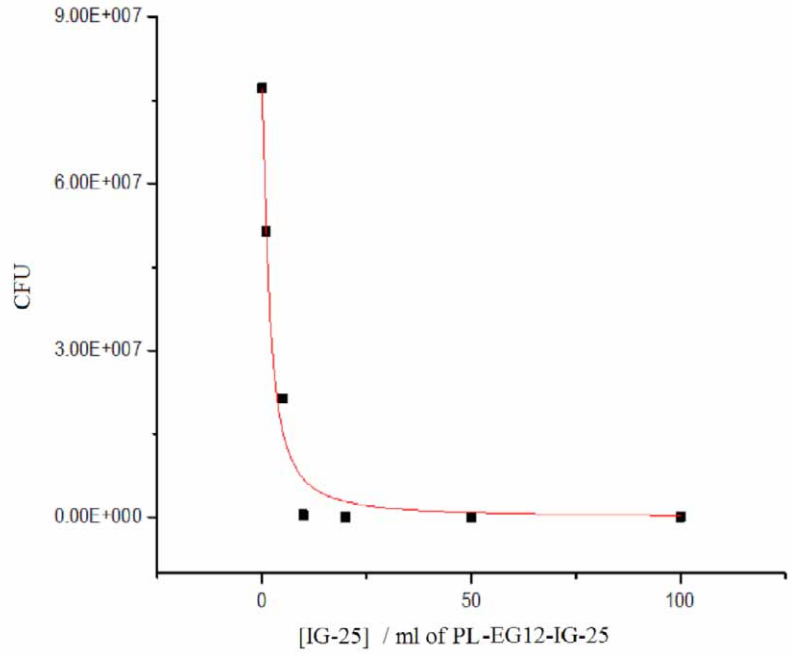


Figure S4. EC_{50} determination for **PL-EG_{12}-IG-25** (CuAAC)

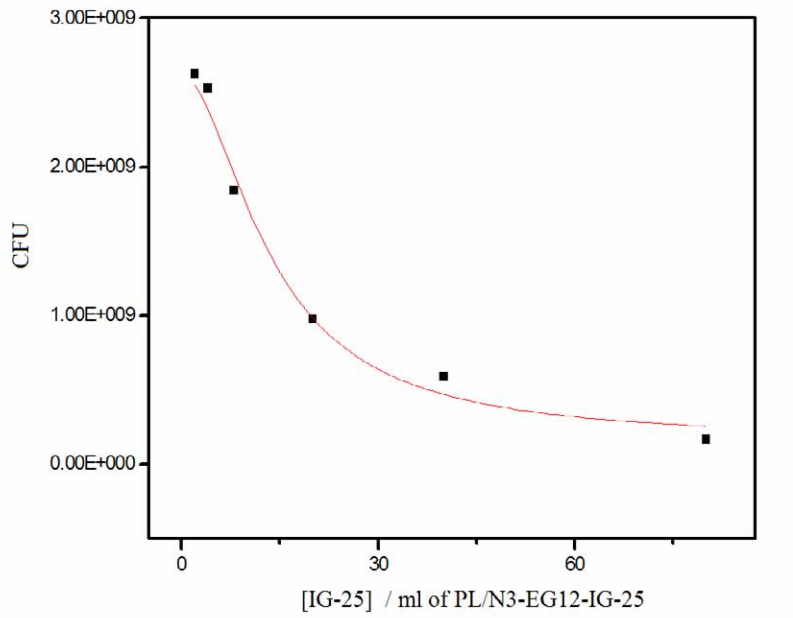


Figure S5. EC_{50} determination for **PL/N_3-EG_{12}-IG-25** (Electrostatic)

Cytotoxicity

To evaluate the cytotoxicity of liposome and IG-25, the cell viability of SV40-HCEC cells was assessed by MTS. The SV40-HCEC cells were cultured in medium DMEM-Ham's F12 (1:1vol/vol) supplemented with 10% FBS, 1% dimethyl sulfoxide (DMSO; Sigma-Aldrich), and 50 μg/mL gentamicin. The culture media was refreshed every 2 to 3 days, and passage at a ratio of 1:3 was performed with routine trypsinization every 4 to 5 days. Based on the results of the anti-bacterial activity of liposome and IG-25, the cells were treated with dosing medium (the experimental medium along with the test compounds) at concentrations of LL-37 20 μg/ml, liposome 500 μg/ml, and **PL-EG$_{12}$-IG-25** 19 μg/ml (amount of IG-25 on the surface of **PL**) for 24 h. The normal culture medium was used as the negative control. Cells were seeded in 96-well plates at an initial concentration of 1,000 cells per well. After 24 h of attachment, the medium was changed to dosing medium containing test compounds for 48 h. After exposure to the MTS solution (Progama), PMS detection reagents were mixed, using a ratio of 20:1 (MTS: PMS), added into the wells and then incubated at 37 °C for 2 h following the manufacturer's instructions. The absorbance was measured at 495 nm with a computer-connected HTS 7000 assay microplate reader (PERKIN ELMER). Results were expressed as OD 495 of each exposure group and the negative control.

Our results showed that the cell growth and cell viability of SV40-HCEC cells was not affected by the liposome or the IG-25 carrying liposome. Compared to the cells treated with 20 μg/ml of LL-37, the liposome and IG-25 did not caused more toxicity to SV40-HCEC cells Take together the anti-bacteria test, the liposome and IG-25 increased the anti-bacteria activity, while not inducing stronger cytotoxicity to SV40-HCEC cells than free LL-37.

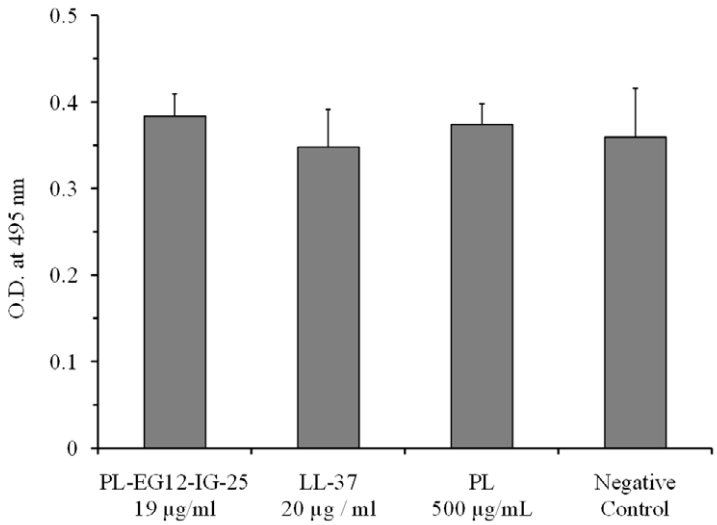


Figure S6. Representative data for cytotoxicity performed on SV40-HCEC cells. The optical density (O.D.) was measured at 495 nm for SV40-HCEC cells, exposed to PL (500 μg/mL), LL-37 (20 μg/mL) and **PL-EG$_{12}$-IG-25** (carrying 19 μg/mL of IG-25 on its

surface) and subsequently treated with MTS reagent. The data indicates that **PL-EG$_{12}$-IG-25** carrying 19 ug/mL of IG-25 on its surface is non toxic to SV40-HCEC cells

References

1. Huang, L. C.; Reins, R. Y.; Gallo, R. L.; McDermott, A. M. *Invest. Ophthalmol. Vis. Sci.* **2007**, *48*, 4498.