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UNIVERSITY OF CALIFORNIA, SAN DIEGO

Nanoparticle Stabilized Liposomes for Acne Therapy

A Thesis submitted in partial satisfaction of the requirements for the degree
Master of Science

in

Nanoengineering

by

Victoria Fu

Committee in charge:

      Professor Liangfang Zhang, Chair
      Professor Michael Heller
      Professor Don Sirbuly

2013

The Thesis of Victoria Fu is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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Chair

University of California, San Diego

2013

DEDICATION

This work is dedicated to each member of the Fu family: Dad, Mom, Kath, Ryan, and Scooter. Despite being so far apart from each other, you all have been able to provide the ample support I needed through my time here at UCSD. You all have truly shown me that love knows no bounds. Thank you for always believing that I could do great things. I couldn't be luckier!

EPIGRAPH

"Far and away the best prize that life has to offer is the chance to work hard at work worth doing."

Theodore Roosevelt

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to research discussions but have also given me a lot of support in completing my research.

Chapter 2, in full, is a reprint of the material as it appears in Advanced Healthcare materials, 2013, Dissaya Pornpattananangkul, Victoria Fu, Soracha Thamphiwatana, Li Zhang, Michael Chen, James Vecchio, Weiwei Gao, Che ming Hu, and Liangfang Zhang. The thesis author was the primary investigator and author of this paper.

Chapter 3, in full, uses the technology of US Provisional Patent PCT/US2011/028014 (2011)¸ Dissaya Pornpattananangkul and Liangfang Zhang, and is a future manuscript submission to ACS Nano, 2014, Victoria Fu, Soracha Thamphiwatana, Weiwei Gao, and Liangfang Zhang. The thesis author was the primary investigator of this manuscript.

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1. Clawson, C.; Ton, L.; Aryal, S.; Fu, V.; Esener, S.; Zhang, L.; "Synthesis and characterization of lipid-polymer hybrid nanoparticles with pH-triggered PEG shedding". *Langmuir,* **2011.** 27, 10556-10561.

2. Aryal, S.; Hu, C-M.; Fu, V.; Zhang, L.; "Nanoparticle drug delivery enhances the cytotoxicity of hydrophobic-hydrophilic drug conjugates", *Journal of Materials Chemistry,* **2012.** 22,994-999.

3. Obonyo, M.; Zhang, L.; Thamphiwatana, S.; Pornpattananagkul, D.; Fu, V.; Zhang, L.; "Antibacterial activities of liposomal linolenic acids against antibiotic-resistant Helicobacter pylori", *Molecular Pharmaceutics*, **2012.** 2677-2685.

4. Pornpattananangkul, D.; Fu, V.; Thamphiwatana, S.; Zhang, L.; Chen, M.; Vecchio, J.; Gao, W.; Huang, C-M. Zhang, L.; In vivo treatment of Propionibacterium acnes infection with liposomal lauric acids". *Advanced Healthcare Materials,* **2013.**

FIELDS OF STUDY

Major Field: Nanoengineering-Nanomedicine

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ABSTRACT OF THE THESIS

Nanoparticle Stabilized Liposomes for Acne Therapy

by

Victoria Fu

Master of Science in Nanoengineering

University of California, San Diego 2013

Professor Liangfang Zhang, Chair

Acne vulgaris is a common skin disease that affects over 40 million people in the United States alone. The main cause of acne vulgaris is *Propionibacterium acnes* (*P. acnes*), resides deep in the pores and follicles of the skin in order to feed on oil produced by the sebaceous glands. The liposome is a lipid based nanoparticle with numerous advantages over free drug molecules as an acne treatment alternative. Bare liposomes loaded with lauric acid (LipoLA) were found to show strong antimicrobial activity

against *P. acnes* while generating minimal toxicity. However, the platform is limited by the spontaneous tendency of liposomes to fuse with each other. Attaching nanoparticles to the surface of liposomes can overcome this challenge by providing steric repulsion and reduce surface tension. Thus, carboxyl-functionalized gold nanoparticles (AuC) were attached to the surface of liposomes (AuC-liposomes) loaded with doxycycline, a general tetracycline antibiotic. These particles were found to have a diameter of 120 nm and a zeta potential of 20.0 mV. Both fluorescent and antimicrobial studies demonstrated that based on electrostatic interaction, negatively charged AuC attached to the liposome's positively charged surface and stabilized liposomes in a neutral pH environment (pH = 7.4). Upon entering the skin's acidic environment (pH = 4), AuC detached from the liposome's surface and liposomes could fuse with *P.acnes* residing in the pores. Furthermore, toxicity studies showed that AuC-liposomes did not induce any significant toxicity, while two of the leading over-the-counter therapies, benzoyl peroxide and salicylic acid, generated substantial skin irritation.

Chapter 1. Liposomes for Acne Therapy

1.1 - An Introduction to Acne

Acne vulgaris is one of the most common skin infections that affects over 80% of the population. Although not a life threatening disease, acne vulgaris can be a frustrating infection that can lower an individual's self-esteem and has been a major cause of suicide ideation in adolescence. Furthermore, the skin damage caused by acne vulgaris also makes the affected tissue more susceptible to other harmful, opportunistic bacteria such as Staphylococcus aureus, and is the leading cause of several eye diseases such as, blepharitis and endophthalmitis.[1] Despite the plethora of treatments available, there is still no cure to acne, and as a result the global acne market is estimated to reach revenues

of \$3.02 billion by 2016.[2] Thus, this infection's pervasiveness makes this infection an ideal model for not only creating an effective nanoparticle solution but a potential therapy solution for a variety of skin infections

There are many factors that are responsible for causing acne vulgaris such as genes, hormonal imbalance, diet, stress, lifestyle, etc. However, the development of the acne lesion is due to the interplay between the bacteria, *Propionibacterium acnes (P. acnes)* and the skin. Integrated within the pores and follicles of the skin, are the sebaceous glands, which are responsible for secreting sebum. Sebum is an oil lubricant that serves as a waterproof shield to maintain the integrity of the skin and prevent the skin from aging. *P. acnes* is an anaerobic, gram-positive bacteria that resides within the follicles and pores to feed on the sebum. This bacteria is normally part of the healthy skin flora, however once the pore or follicle is compromised, the overgrowth of bacteria will cause the acne infection to occur. In order to breakdown sebum, P. acnes must secrete a digestive enzyme which can also break down the surrounding tissue. The rupturing of the follicle walls results in the host immune cells to react with P. acnes, leading to inflammatory acne. Severely inflamed acne lesions can cause hyper-pigmentation and permanent skin scarring.[3]

Many antimicrobial agents have been developed for acne treatment including adapalene, tazarotene, erythromycin, clindamycin, benzoyl peroxide (BPO), salicylic acid, etc. While these existing anti-acne agents are effective, they usually produce considerable side effects. Table 1.1 is a list of the top five drugs used for acne treatment and their associated side effects.

Table 1.1 – The most widely used acne therapies and their associated side effects[4]

Top Acne Drugs	Side Effects
Benzoyl Peroxide	Redness, rash, burn, sun sensitivity
Salicylic Acid	Redness, rash
Oral antibiotics	Disruption of intestinal microflora, antibiotic resistance
Retinoids	Redness, rash, burn, sun sensitivity
Isotretinoin	Birth defects, depression, blurred vision

BPO has been one of the most frequently used topical mediciation for acne treatment; however, it produces a high incidence of erythema, scaling, burning, and hair bleaching. Oral antibiotics although highly effective, often pose the risk of harming the intestinal microflora and creating antibiotic-resistant P. acnes. For instance the use of isotretinoin, a vitamin A-derivative retinoid prescribed for systemic treatment of severe acne, is strictly regulated and not applicable for most acne patients owing to its strong teratogenicity.[4] Thus, it is necessary to redesign anti-acne agents in order to provide both excellent therapeutic efficacy and negligible adverse side effects.

1.2 - The Advantages of using Liposomes as an Acne Therapy

Nanotechnology has become an expanding field of interest for antimicrobial drug delivery because of the many added advantages nanotechnology can provide. By delivering drugs using nano-sized platforms, we have the ability to control drug release, prolong drug circulation time, and deliver multiple agents, which improves the overall effectiveness of the drug.

In 1964, Alec Bangham and Robert H. Horne decided to observe the interaction of phospholipids in water using an electron microscope. The resulting formation of "bag-like" structures within the aqueous environment was marked as the initial discovery of the liposome platform.[5] Liposomes are spherical vesicles that can be characterized by their lipid bilayer structure, which is composed of amphiphilic lipid molecules. The amphiphilic lipid's structure contains a hydrophilic head and a hydrophobic tail. This allows liposomes to spontaneously assemble in water, exposing their hydrophilic head group and protecting the hydrophobic tail within a bilayer.[6] It is because of this formed bilayer structure that liposomes can be loaded with drugs of varying hydrophobicity. As shown in Figure 1.1, hydrophilic drugs can be loaded within the interior compartment, while hydrophobic drugs can be loaded within the lipid bilayer. Additionally, amphiphilic molecules such as fatty acids can be incorporated within the liposome's lipid membrane.
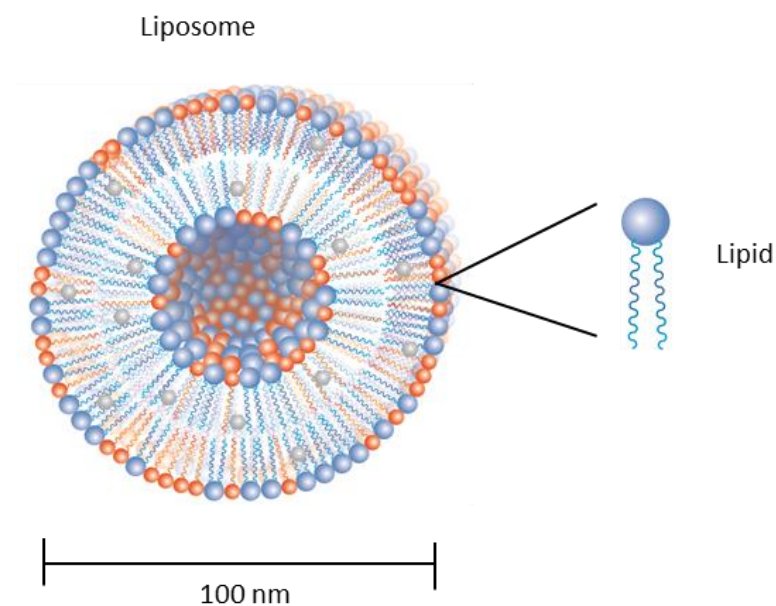
Figure 1.1 - A breakdown of the liposome platform. Liposomes are composed of lipid molecules that have a hydrophilic head group and a hydrophobic tail. Hydrophobic drug can be loaded within the interior of the lipid bilayer. Hydrophilic drug molecules are encapsulated within the inner core of the liposome. Amphiphilic molecules such as fluorescent lipids or fatty acids can be incorporated into the lipid bilayer.

By encapsulating drug, free drug molecules are protected from the external environment, reducing drug degradation and potential adverse side effects. The liposome's surface can also be easily functionalized with specific targeting moieties in order to deliver liposomes and their drug cargo to the specific site of interest. This platform can be fine-tuned to meet specific goals by modifying the composition, size, surface charge, and other formulation properties.

Liposomes can be synthesized with either natural or synthetic lipids. The most common lipid used is phosphotidylcholine, which can be extracted from egg yolk or soy beans. Lipid interaction within the bilayer is governed by phase transition temperature. This is the temperature at which lipids transition from an ordered gel phase to a disordered liquid crystalline phase. In the ordered gel phase, hydrocarbon chains are fully extended, closely packed, and as a result, reduced the mobility of lipids within the bilayer. During the disordered liquid crystalline phase, lipids are mobile due to the random orientation of the hydrocarbon chains. This phenomenon is associated with the length of the lipid's tail, which governs how strong the lipid's Van der Waals interaction is between adjacent lipids. Thus, using lipids with longer tails will give the assembled liposome a more rigid structure since the lipid's mobility has been restricted.[7]

Cholesterol is also usually incorporated into the liposome formulation in order to give the liposome membrane rigidity and stability. Liposomes that are made completely from unsaturated lipids form crimps within the lipid chains which can cause problematic drug leakage. By incorporating cholesterol into the lipid membrane, the spaces within the

chain are occupied, decreasing the flexibility of the chains and making the lipid membrane more inflexible.[8]

After 50 years of liposome research, liposomes have become one of the most widely established drug carriers for delivering a variety of cosmetic, pharmaceutical, and imaging agents. Fifty years later, liposomes have become one of the most established drug delivery carriers, with 12 drugs clinically approved and 22 more liposome technologies in clinical trial.[9] A list of approved liposome formulations as of 2012 has been listed below in Table 1.2.

Table 1.2 - A list of clinically approved liposome formulation[9]

Product Name	Route of Injection	Drug	Particle type/size	Drug form/Storage time	Approved indication
Ambisome	Intravenous	Amphotericin B	Liposome	Powder/36 months	Severe fungal infection
Abelcet	Intravenous	Amphotericin B	Lipid complex	Suspension/24 months	Severe fungal infections
Amphotec	Intravenous	Amphotericin B	Lipid complex	Powder/24 months	Severe fungal infection
DaunoXome	Intravenous	Daunorubicin	Liposome	Emulsion/12 months	Blood tumors
Doxil	Intravenous	Doxorubicin	PEGylated liposome	Suspension/20 months	Kaposi's sarcoma, Ovarian/breast cancer
Lipo-dox	Intravenous	Doxorubicin	PEGylated	Suspension/36 months	Kaposi's sarcoma, ovarian/breast cancer
Myocet	Intravenous	Doxorubicin	Liposome	Powder/18 months	Combination therapy with cyclophosphamide in metastatic breast cancer
Visudyne	Intravenous	Verteporfin	Liposome	Powder/48 months	Age-related molecular degeneration, pathologic myopia, ocular histoplasmosis

Table 1.2 - continued

Product Name	Route of Injection	Drug	Particle type/size	Drug form/Storage time	Approved indication
Depocyt	Spinal	Cytarabine	Liposome	Suspension/18 months	Neoplastic meningitis and lymphomatous meningitis
DepoDur	Epidural	Morphine sulfate	Liposome	Suspension/24 months	Pain management
Epaxal	Intramuscular	Inactivated hepatitis A virus (strain RG-SB)	Liposome	Suspension/36 months	Hepatitis A
Inflexal V	Intramuscular	Inactivated hemaglutinine of Influenza virus strains A and B	Liposome	Suspension/12 months	Influenza

Abbreviations: DOPE, dioleoylphosphatidylethanolamine; DOPC, dioleoylphosphatidylcholine; DPPG, dipalmitoylphosphatidylglycerol; HSPG, hydrogenated soy phosphatidylcholine; DSPG, distearoylphosphatidylglycerol; EPC, egg phosphatidylcholine; DSPC, distearoylphosphatidylcholine; DMPC, 1-α-dimyristoylphosphatidylcholine; DMPG, l-α-dimyristoylphosphatidylglycerol; EPG, egg phosphatidylglycerol; PEG 2000-DSPE, polyethylene glycol 2000-distearoyl-phosphatidylethanolamine.

The liposome's bilayer gives this platform two unique advantages. The first advantage is the ability to fuse with other cells and pathogenic bacteria, making this nanotechnology ideal for bacterial infection treatments. By fusing with bacteria, liposomes are able to disrupt the bacteria membrane and deliver a concentrated amount of drug cargo into the interior of the bacteria for effective eradication. The platform has high biocompatibility because the liposome membrane mimics the lipid bilayer of a cell membrane. Specifically for the treatment of P. acnes, the liposome's sub-100 nm size, will allow liposomes to transport drug through the skin, reach the follicle site, and deliver drug to the targeted site. Furthermore, having liposomes fuse with P. acnes and directly deliver the drug cargo into the interior of the bacteria should induce minimal toxicity with

the maximum efficacy. Because of these numerous advantages, the liposome platform can be a promising solution for creating a safe and effective acne therapy.[10]

Chapter 2. Lauric Acid Liposomes for Acne Therapy

2.1 - Introduction

Acne infection is a common skin disorder that afflicts up to 80% of individuals in their lives.[11] Acne develops as a result of blockages in follicles due to the over-production of sebum by sebaceous glands. The accumulation of sebum triggers the overgrowth of *Propionibacterium acnes (P. acnes),* a Gram-positive anaerobic bacterium strongly associated with acne infection.[12] Upon the rupture of follicle walls, the host immune cells react with *P. acnes*, leading to inflammatory acne.[12-14] Severely inflamed acne lesions can cause hyper-pigmentation and permanent skin scarring, which can be a source of embarrassment, stress, and low self-esteem among young people, therefore affecting their mental health and psychological development.[15] Many antimicrobial

agents have been developed for acne treatment including adapalene, tazarotene, erythromycin, clindamycin, benzoyl peroxide (BPO) and other antibiotics.[16-18] While these existing anti-acne agents are effective, they usually produce considerable side effects. For instance, BPO has been one of the most frequently used epicutaneous medications for acne treatment; however it produces a high incidence of erythema, scaling, burning and hair bleaching.[19-20] Oral antibiotics, although highly effective, often pose the risk of harming the intestinal microflora and creating antibiotic-resistant *P. acnes.* For instance, the use of isotretinoin, a vitamin A-derived retinoid prescribed for systemic treatment of severe acne, is strictly regulated and not applicable for most acne patients owing to it strong teratogenicity.[21] Clearly, new anti-acne agents with both excellent therapeutic efficacy and negligible adverse side effects are highly desirable.

Free fatty acids (FFAs) have been known to be responsible for part of the self-antimicrobial disinfecting activity of the human skin against microbial colonization, and their bactericidal properties have been extensively investigated.[22-25] These lipid-like molecules are ubiquitous and natural, which are expected to be less harmful than the synthetic antimicrobial molecules. Various FFAs have been disclosed to possess potent antimicrobial activities against a diverse range of bacteria.[26] For example, oleic acid has been reported to inhibit the growth of *Staphylococcus aureus* in a mouse model,[27,28] and lenolenic acid is able to overcome the antibiotic resistance developed by various clinically isolated strains of *Helicobacter pylori.*[29,30] While the antimicrobial working mechanism of FFAs is yet fully understood, accumulating evidence suggests that they are effective in disrupting bacterial membranes and increasing membrane permeability.[26]

Due to the low water solubility of FFAs, they usually need to be formulated together with other excipient materials to prepare stable and water-soluble formulations.[31] Among various FFA formulations, liposomal FFAs represent a robust and popular platform.[27,29,32] The amphiphilic nature of FFAs allows them to be readily incorporated into the lipid bilayer of liposomes with a high loading efficiency. The resulting liposomal formulation not only enhances the water-solubility of FFAs but also protects them from degradation in physiological conditions. Moreover, the liposomal formulation facilitates the interaction of FFAs with bacterial membranes and thus improves their antimicrobial effectiveness.[33]
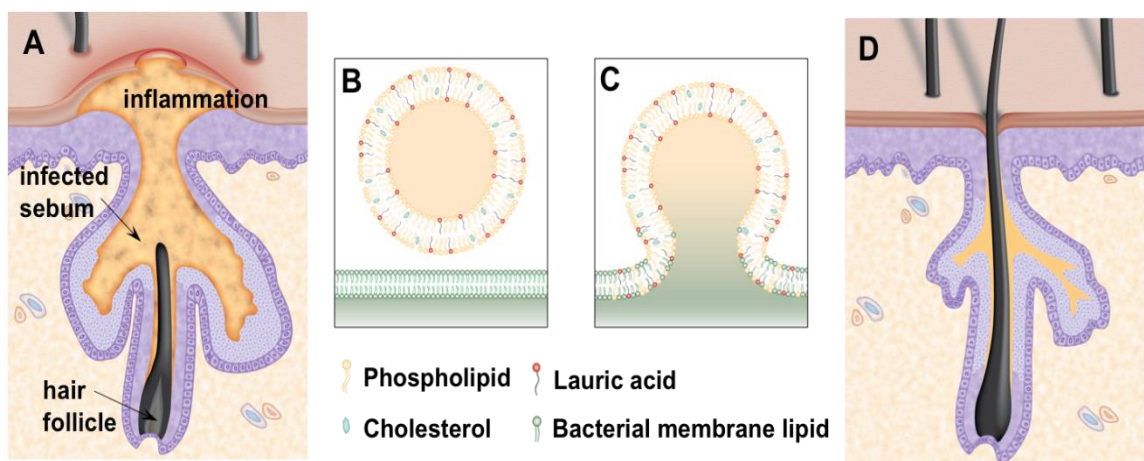
Figure 2.1 - Schematics of using liposomal lauric acids (LipoLA) to treat acne infection caused by *Propionibacterium acnes* (*P. acnes*) bacteria. (A) Anatomic illustration of skin with inflammatory acne. (B) Structure and composition of LipoLA consisting of phospholipid, cholesterol and lauric acid. (C) Hypothesized mechanism of action; LipoLA fusing into bacterial membranes. (D) Anatomic illustration of skin after LipoLA treatment.

We have previously reported that lauric acids had strong antibacterial activity against *P. acnes* without obvious cytotoxicity against SZ95 human sebocytes.[34] We have also shown that the bactericidal activity of lauric acids against *P. acnes* was well

preserved after loading them to a liposome formulation.[32] Herein, we systematically evaluate the *in vivo* therapeutic efficacy and toxicity profile of liposomal lauric acids (LipoLA) for the treatment of acne infection caused by *P. acnes* bacteria. Using a mouse ear model, we test the bactericidal property of LipoLA against *P. acnes* through two administration routes, intradermal injection and topical application. Skin toxicity of LipoLA is thoroughly evaluated in comparison with two most popular over-the-counter acne care drugs, BPO and salicylic acid. The findings from this study provide more clinically related assessments of LipoLA as a new, effective, and safe anti-acne medication (**Figure 2.1**).

2.2 - Experimental Methods

2.2.1 - ***Materials:*** Hydrogenated L-a-phosphatidylcholine (Egg PC) and cholesterol were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). Lauric acic, hydroxyethyl cellulose (HEC), and glycerin were obtained from Sigma Aldrich (St Louis, MO). Brucella broth, Gas-Pak, and agar were purchased from BD (Sparks, MD). Reinforced clostridial medium was purchase from Oxoid (Hampshire, UK). Institute of Cancer Research (ICR) mice were purchased from Charles River (Wilmington, MA). Polyethylene glycol 400 (PEG 400) was purchased from USB Corporation (Cleveland, OH). Commercial Nair® cream, 10% benzoyl peroxide (Clean & Clear®), and 2% salicylic acid (Clean & Clear®) were purchased from a local drug store.

2.2.2 **- *Preparation of bacteria:*** *P. acnes* (ATCC 6919) (American Type Culture Collection, Manassas, VA) was cultured on a reinforced clostridial medium (RCM) agar plate, under anaerobic condition at 37°C for 3 days. A single colony was then inoculated

in RCM and cultured at 37°C till reaching approximately OD_{600}=1.5 (logarithmic growth phase) under anaerobic condition. Note that for *P. acnes*, $OD_{600} = 1$ is corresponding to a bacterial concentration of 5×10^{8} CFU/mL. The bacteria were harvested by centrifugation at 13,000 rpm for 3 min, washed with PBS, repeated 3 times, and then suspended to appropriate amount of PBS for experiment uses.

2.2.3 - ***Preparation of LipoLA and LipoLA gel:*** LipoLA were prepared by a vesicle extrusion technique as previously reported [22]. In brief, lipid solution composed of Egg PC, cholesterol, and lauric acid (5:1:4, weight ratio) was dissolved in chloroform and evaporated under nitrogen gas. The dried lipid film was then rehydrated with sterile PBS buffer. The suspension of lipids was vortexed and then sonicated in a bath sonicator (Fisher Scientific FS30D) to produce multilamellar vesicles (MLV). The acquired MLV were further sonicated by a Ti-probe (Branson 450 sonifier) to produce small unilamellar vesicles (SUV). Lastly, the resulting SUV were extruded through a 100 nm pore-sized polycarbonate membrane to form the final product of LipoLA. The size and zeta potential of the resulting LipoLA were determined using the Malvern Zetasizer ZS (Malvern Instruments, UK), from which the mean diameter of LipoLA was measured through dynamic light scattering (DLS), and the zeta potential through electrophoretic mobility measurements. All characterization measurements were repeated three times at 25°C.

To prepare LipoLA cellulose gel, the mixture of HEC, glycerin, and PEG 400 (7.0, 5.6, and 1.8 wt%) were first swelled by 50 vol% of PBS under stirring for 15 min on a 60°C hot plate. The gel was then stirred continuously for 24 h at room

temperature. Next, the pre-swelled gel was mixed with 50 vol% of LipoLA solution and vortexed until completely homogenized to obtain LipoLA gel.

2.2.4 - *Fusion study between LipoLA and P. acnes:* Förster resonance energy transfer (FRET) study was performed to investigate the interaction between LipoLA and *P. acnes*. A fluorescent donor C_6NBD (0.1 mol%) and a fluorescent acceptor DMPE-RhB (0.5 mol%) were concurrently incorporated into the bilayer membrane of LipoLA (0.5 mg/mL) by mixing the dyes with other lipid components prior to the preparation of LipoLA. The FRET-pair labeled LipoLA were then incubated with *P. acnes* at concentrations of 1×10^8, 1×10^9, 3.3×10^9, 6.6×10^9, and 1×10^{10} CFU/mL respectively. After 30 min of incubation at room temperature, the excess LipoLA were removed by centrifugation. The bacterial suspensions were excited at 470 nm and the fluorescence emission spectrum of C_6NBD was recorded using a fluorescent spectrophotometer (BioTek Instrument, USA). Florescence intensity of all samples was subtracted with background, which was the fluorescence intensity of *P. acnes* solution at the corresponding bacterial concentrations. FRET-pair labeled LipoLA without *P. acnes* was used as a negative control.

2.2.5 - *In vitro antimicrobial activity of LipoLA against P. acnes and bacterial morphology study:* The antimicrobial activity of LipoLA against *P. acnes* was determined by incubating *P. acnes* (1×10^7 CFU/mL) with LipoLA (2 mg/mL) in PBS at 37°C under anaerobic condition for 5 h, while *P. acnes* in PBS was used as a negative control. Following incubation, the samples were diluted 1:10 to $1:10^6$ in PBS, and 10 µL

of each sample was spotted on RCM agar plates. The samples were incubated at 37°C under anaerobic condition for 3 days, and then the CFU of *P. acnes* was quantified.

The morphology of *P. acnes* treated by LipoLA was visualized with scanning electron microscope (SEM). *P. acnes* (1×10^8 CFU/mL) were incubated with LipoLA (4 mg/mL) in PBS at 37 °C under anaerobic condition for 5 h. The same amount of *P. acnes* incubated in PBS was used as a negative control. Samples were then fixed with 2% glutaraldehyde in $1\times$ PBS (pH=7.4) at room temperature for 30 min. Post fixing, the samples were centrifuged to remove glutaraldehyde, washed three times with water, and resuspended in 100μL water. Then 5μL of bacterial suspension was dropped onto a polished silicon wafer and allowed to dry overnight in a biosafety cabinet. The samples were then coated with chromium and imaged with an FEI XL30 Environmental SEM.

2.2.6 - *In vivo antimicrobial activity of LipoLA against P. acnes through intradermal injection:* The antimicrobial activity of LipoLA against *P. acnes* in a physiological environment was tested using ICR mouse ears through intradermal injection. Right before injection, LipoLA (with concentrations of 2, 4, 6, and 8 mg/mL, respectively) were mixed with *P. acnes* (1×10^8 CFU/mL). The resulting solution (10 μL) was intradermally injected into the ears of mice. *P. acnes* mixed with BPO (16 mg/mL) in 5% DMSO was used as a positive control and *P. acnes* in PBS buffer was used as a negative control. The ears were collected 24 h after injection using an 8 mm biopsy punch and homogenized in 1 mL of sterile PBS (Mini-Beadbeater™). Homogenates were diluted 1:10 to $1:10^6$ in PBS, and 10 μL of each dilution was spotted on RCM agar plates. Then, the agar plates were incubated at 37°C under anaerobic condition for 3 days, and the CFU of *P. acnes*

was quantified. Ears without *P. acnes* inoculation served as a negative control to ensure that there was no contamination from other bacteria. Six mice were used for each group (n=6) and the experiment was repeated three times for statistical significance.

2.2.7 - ***In vivo therapeutic efficacy of LipoLA against acne infection through topical administration:*** To induce *P. acnes* infection, the surface of the mouse ears was first scratched with a 25G needle tip to generate a wound with an area of around 10 mm^2. Then, 1 μL of *P. acnes* (1x10^9 CFU/mL) was inoculated onto the wound to yield 1×10^6 CFU of *P. acnes* per ear. After 10 min of inoculation, LipoLA cellulose gel was applied topically onto the wound. The LipoLA gel was applied daily for 2 days successively. After 2 days of drug application, the ears were collected, and the same procedure as described in 2.6 was performed to quantify the CFU of *P. acnes* on the ear. Commercial BPO cream and PBS gel were used as a positive and a negative control, respectively. Six mice were used for each group (n=6) and the experiment was repeated three times for statistical significance.

2.2.8 - ***Skin toxicity:*** The skin toxicity of LipoLA was tested on the back skin of ICR mice. Specifically, the back of the mice was shaved 24 h prior to the study, followed by topically administering LipoLA cellulose gel (2 mg/mL). Blank PBS gel (without LipoLA) was used as a negative control. Commercial Nair® cream, 10% PBO cream, and 2% salicylic acid (SA) gel were used as positive controls. After 24 h, the skin morphology was examined and imaged. Skin irritation was scored according to Draize's system.[38] For histological observation, the skin was cross-sectioned by an 8 mm biopsy punch, stained with hematoxylin and eosin (H&E), and imaged with a microscope. Six

mice were used for each group (n=6) and the experiment was repeated three times for statistical significance.

2.3 - Interaction between LipoLA and *P. acnes* bacteria

The building materials of LipoLA are all from natural sources, including hydrogenated L-a-phosphatidylcholine (EggPC) from egg yolk, cholesterol from animal fat, and lauric acid from coconut milk. With a weight ratio of 5:1:4, the mixture of EggPC, cholesterol and lauric acid were prepared to form LipoLA through a common vesicle extrusion method. The resulting LipoLA have an average diameter of 119.9 ± 0.3 nm, a polydispersity index of 0.12, and an average surface zeta potential of -43.8 ± 1.5 mV, measured by DLS. The interaction between the resulting LipoLA and *P. acnes* bacteria were studied by FRET technique. We first incorporated 0.1 mol% of fluorescent donor C_6NBD (excitation/emission = 470/520 nm) and 0.5 mol% of fluorescent acceptor DMPE-RhB (excitation/emission = 550/580 nm) into the lipid bilayer of LipoLA to prepare FRET-pair labeled LipoLA. At the used molar concentrations of the donor and the acceptor, the fluorescence emission from the donor was maximally quenched by the acceptor through a nonradiative long-range dipole-dipole coupling mechanism.

By mixing the FRET-pair labeled LipoLA (0.5 mg/mL) with *P. acnes* at different bacterial concentrations ranging from 1×10^8 to 1×10^{10} CFU/mL for 30 min, we observed increasing emission intensity of C_6NBD at 520 nm when the samples were excited at the wavelength of 470 nm (**Figure 2.2**). The rise in the emission peak of the fluorescent donor indicates the fusion of LipoLA with bacterial membranes, which causes an

increase in spatial separation between the two dyes and the fluorescence recovery of the

donor. Note that the emission of DMPE-RhB at 580 nm was not selected for comparison
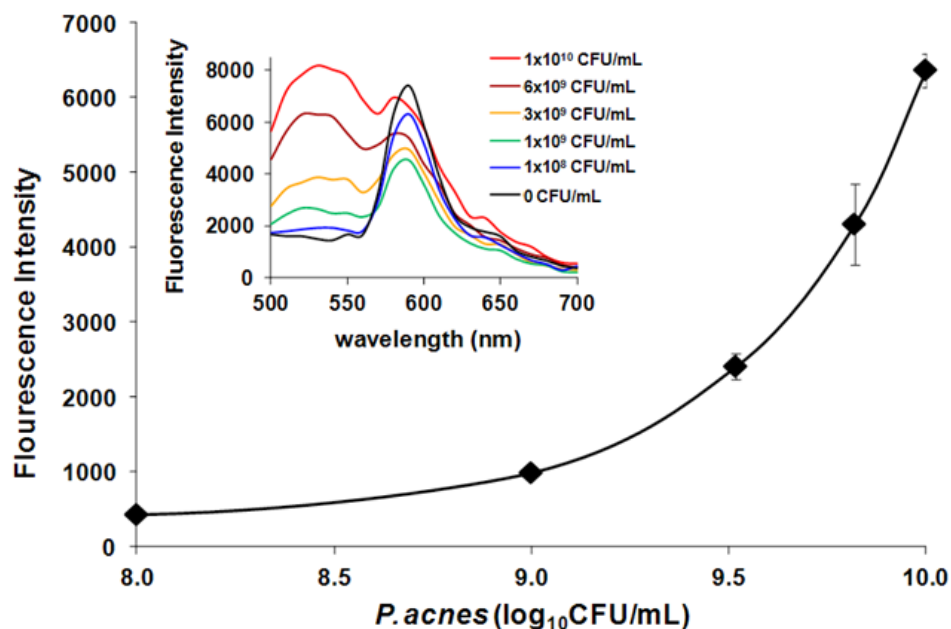
Figure 2.2 - FRET measurements of the fusion between LipoLA and *P. acnes* bacteria. LipoLA were labeled with both a fluorescent donor (C_6NBD) and a fluorescent acceptor (DMPE-RhB) at a proper molar ratio that the acceptor maximally quenched the fluorescence emission from the donor. The FRET-pair labeled LipoLA were then incubated with *P. acnes* at various bacterial concentrations. After removing the excess LipoLA, all samples were excited at 470 nm and the fluorescence emission spectra were recorded (Inset). A rise in emission intensity of C_6NBD at 520 nm was observed, indicating the occurrence of spatial separation of C_6NBD and DMPE-RhB due to the fusion between LipoLA and bacterial membranes.

because DMPE-RhB dye could be excited by not only the FRET from C_6NBD but also

the excitation wavelength at 470 nm, making it difficult to make an accurate comparison.

2.4 - *In vitro* antimicrobial activity and bacterial morphology
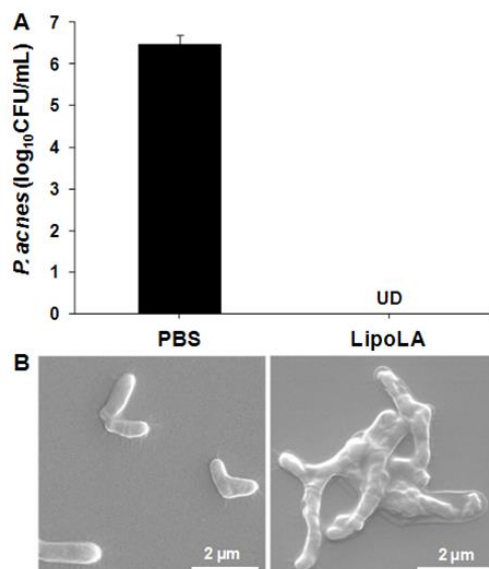
Figure 2.3 - *In vitro* antimicrobial activity of LipoLA against *P. acnes* and morphology of *P. acnes* after LipoLA treatment. LipoLA were incubated with *P. acnes* for 5 h under anaerobic condition. (A) *P. acnes* bacteria were diluted and spotted on agar plates, and the bacterial number was quantified. LipoLA completely killed the bacteria. PBS was used as a negative control. Data represents mean ± SD of three independent experiments. UD: undetectable. (B) Following LipoLA or PBS treatment, the bacteria were fixed and imaged by scanning electron microscope (SEM). A destruction of bacterial membranes and an absence of fimbriae were observed with the LipoLA treated sample (right panel) as compared to the PBS treated sample (left panel).

To test the antimicrobial activity of LipoLA against *P. acnes in vitro*, LipoLA (2 mg/mL) were incubated with *P. acnes* (1×10^7 CFU/mL) at 37°C for 5 h under anaerobic condition. The results showed that LipoLA completely killed *P. acnes* without detectable CFU formed on RCM agar plates while the amount of *P. acnes* incubated with PBS buffer (negative control) was 6.5×10^6 CFU/mL (**Figure 2.3A**). After quantifying the *in vitro* antimicrobial activity of LipoLA against *P. acnes* bacteria, we next investigated the effect of LipoLA on the morphology of the bacteria using SEM. *P. acnes* bacteria were incubated with LipoLA for 5 h, fixed with 2% glutaraldehyde, and then observed by

SEM. As shown in **Figure 2.3B**, the SEM micrograph of untreated sample (i.e., incubated with PBS buffer) showed that *P. acnes* has a regular rod-like structure with a smooth surface and fimbriae around the organism. In contrast, bacteria treated with LipoLA exhibit clear abnormality; the bacterial surface was irregularly deformed and shrunk with the absence of the fimbriae. These results indicate that the interaction of *P. acnes* bacteria with LipoLA disrupts the bacterial membrane structure, suggesting a possible mechanism by which LipoLA kill the bacteria. This finding is consistent with previous report of structural change of *Clostridium perfringens*, another anaerobic Gram-positive bacterium, upon the treatment of FFAs.[35]

2.5 - *In vivo* antimicrobial activity

Next we tested the *in vivo* antimicrobial activity of LipoLA against *P. acnes* using an ICR mouse ear model via intradermal injection. Mouse ear was selected for this study because its confined structure can retain all inoculated bacteria at the injection area. To evaluate the antimicrobial activity of LipoLA against *P. acnes* in physiological environment, LipoLA (2, 4, 6, or 8 mg/mL) were mixed with *P. acnes* (10^8 CFU/mL) right before the injection. Subsequently, 10 μL of the mixture solution was intradermally injected into the mouse ear. After 24 h of injection, the ear was collected using an 8-mm biopsy punch and homogenized to quantify the remaining amount of *P. acnes.* In this study, BPO at a concentration of 16 mg/mL in 5% dimethyl sulfoxide (DMSO) premixed with *P. acnes* (10^8 CFU/mL) was used as a positive control, *P. acnes* in PBS was used as a negative control, and the injection of PBS without *P. acnes* was used to demonstrate that there was no contamination from other bacteria during the experiments.
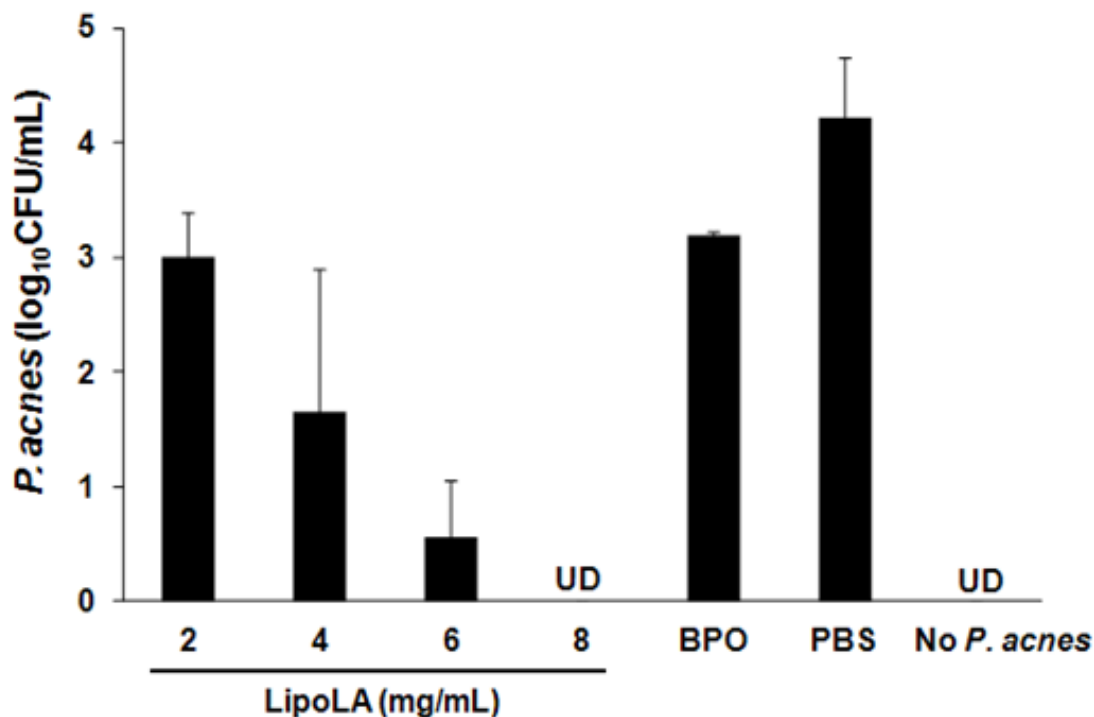
Figure 2.4 - *In vivo* antimicrobial activity of LipoLA against *P. acnes* through intradermal injection. Different concentrations of LipoLA (2, 4, 6, or 8 mg/mL) were mixed with *P. acnes* and immediately injected into mouse ears (n=6 per group), followed with bacteria quantification at 24 h. BPO (16 mg/mL) was used as a positive control. PBS and none *P. acnes* inoculation were served as negative controls. Data represents mean ± SD of three independent experiments. UD: undetectable.

As shown in **Figure 2.4**, significant reductions of *P. acnes* were observed when treated with LipoLA. The measured CFU of *P.acnes* were 1.0×10^3, 4.4×10^1, 3.6, and 0 CFU/mL for 2, 4, 6, and 8 mg/mL of LipoLA, respectively. In contrast, the detected bacterial number for the negative control group (treated with PBS buffer) was 1.7×10^4 CFU/mL and the positive control (treated with BPO) reduced the bacterial number to 1.6×10^3 CFU/mL. The control group of mice injected with only PBS (without *P. acnes*) had no detectable bacteria, confirming no bacterial contamination during the experiments. These results confirm that LipoLA can effectively kill *P. acnes* bacteria in physiological environment such as in the dermis.

2.6 - *In vivo* therapeutic efficacy

To further evaluate the potential of LipoLA as an effective anti-acne agent, we next developed a topical model of *P. acnes*-induced inflamed acne infection and tested the efficacy of LipoLA against *P. acnes*. In the case of inflammatory acne lesion in human, chemotactic factors are secreted from *P. acnes*, which attract inflammatory cells into the sebaceous follicle. These cells further release other inflammatory factors, such as lysosomal enzymes, proteases, and reactive oxygen that damage follicle wall, leading to inflammatory lesions.[36] Our previous study showed that by inoculating *P. acnes* into the ear of ICR mouse, macrophages were attracted into the injection site, similar to *P. acnes*-induced inflammatory acne lesions in human.[34] Herein, 1×10^6 CFU of *P. acnes* were inoculated on the ear of the mouse that has pre-scratched wound with an area of around 10 mm^2 to generate *P. acnes*-inducted inflammation. Following the inoculation, 2 mg/mL of LipoLA prepared in a water-based gel was applied daily onto the wound for 2 days. The gel was composed of hydroxyethyl cellulose (7.0 wt%), glycerin (5.6 wt%), and polyethylene glycol 400 (1.8 wt%). As shown in **Figure 2.5**, the 2 mg/mL LipoLA in a gel form completely killed the inoculated *P. acnes* bacteria. The LipoLA gel shows a comparable efficacy to the positive control of 10 wt% BPO cream purchased from a local drug store. The detected bacterial number for the negative control group (treated with PBS buffer) was 1.0×10^4 CFU/mL, reduced from the initially inoculated number of 1.0×10^6 CFU/mL. This reduction might be attributed to the clearance from the host's immune system and tissue loss during the grinding process. The control group of mice without *P. acnes* inoculation had no detectable bacteria, indicating that the study groups were free from contamination of normal flora. Commercialized BPO cream has been

considered as the standard treatment of mild to moderate acne due to its effective antimicrobial and oxidizing activity since the 1960s.[27] However, due to BPO's common side effects, such as dryness, irritation, and burning, it has resulted in lowered patient compliance and unsuccessful treatment. The fact that LipoLA gel could achieve a comparable efficacy to BPO cream suggests that LipoLA may become a favorable alternative for acne treatment.
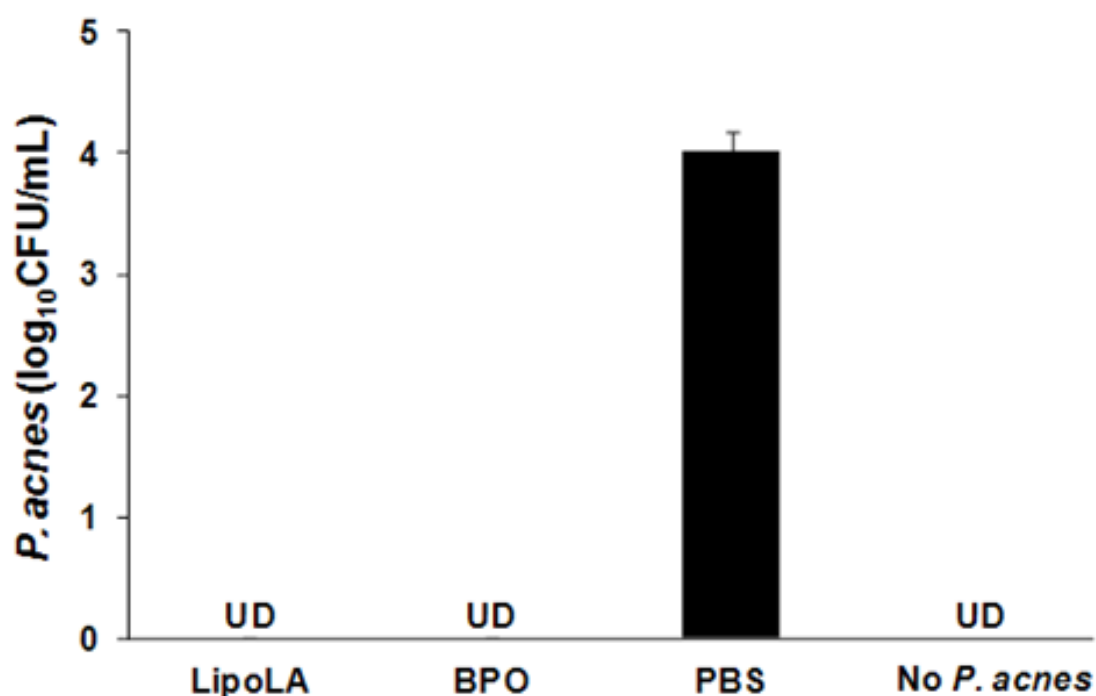
Figure 2.5 - *In vivo* therapeutic efficacy of LipoLA for the treatment of *P. acnes* infection through topical administration. *P. acnes* (10^6 CFU) were inoculated onto a wound generated on a mouse ear (n=6 per group), followed with topical application of LipoLA gel daily for 2 days successively. At 72 h, the ear was collected, and the remaining amount of *P. acnes* was quantified. Commercial BPO cream from a drug store was used as a positive control. PBS gel and none *P. acnes* inoculation were served as negative controls. Data represents mean ± SD of three independent experiments. UD: undetectable.

2.7 - Toxicity of LipoLA to normal skin tissues

The *in vivo* toxicity profile of LipoLA was evaluated by examining the changes of skin morphology after topically applying LipoLA gel onto the back skin of ICR mice. The toxicity of LipoLA was compared with 10 wt% BPO, 2 wt% salicylic acid (SA), and Nair[®] hair removal cream. In this study, the mouse back skin was shaved 24 h prior to drug administration to allow skin to recover from any possible disturbance to the stratum corneum [38] and then moistened with PBS right before the experiments. Then the samples were topically applied onto the skin and left for 24 h, followed by removing the drugs and moistening with PBS.
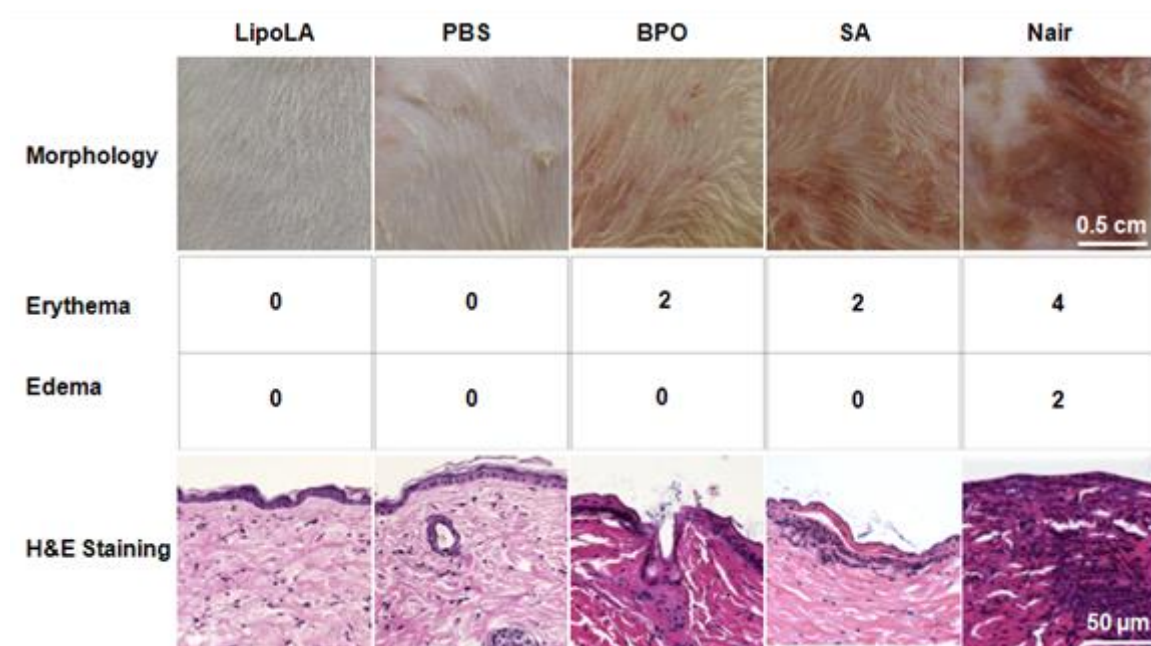
Figure 2.6 - Toxicity study of LipoLA on mouse back skin. LipoLA gel was topically applied onto shaved mouse back skin (n=6 per group). After 24 h, the gel was removed and the skin was analyzed. Blank PBS gel was used as a negative control. BPO, salicylic acid (SA), and Nair[®] cream were used as positive controls. (A) Morphology of the skin after treatment imaged by a camera. (B) Draize's irritation scores indicating erythema. (C) Edema scores of the skin. (D) Back skin was cross-sectioned, stained with hematoxylin and eosin (H&E), and viewed by a microscope. Data is representative of three separate experiments with similar results.

As shown in Figure 6, the skin treated with LipoLA gel maintained its normal structure and no erythema or edema was observed. This skin structure was similar to that treated with blank PBS gel, which served as a negative control. In contrast, both BPO cream and SA gel caused moderate erythema, but no edema was observed. Nair® cream was the most irritating compound, which caused severe erythema and moderate edema throughout the application area. According to the Draize's irritation scoring system, the erythema and edema scores of LipoLA gel were 0 and 0, respectively, indicating no apparent irritation. For both BPO cream and SA gel samples, the scores were similar, which are 2 and 0 for the erythema and edema respectively, representing well defined erythema throughout the skin. In the case of Nair® cream, an erythema score of 4 indicated severe erythema with beet redness and slight eschar formation, which signified in depth injury. The average edema score from 3 Nair® cream treated mice was 2, demonstrating moderate edema with raised edges of the affected area. The *in vivo* toxicity of LipoLLA was further evaluated by examining the histology of the treated skin. The skin biopsy was collected and stained with hematoxylin and eosin (H&E). As shown in **Figure 2.6** (bottom row), the LipoLA treated samples showed an undisturbed skin structure with a layer of healthy epidermal cells on top of the dermis layer, which was identical to the PBS treated skin samples. In contrast, for both BPO and SA treated samples, the epidermis layer was destroyed and disconnected and there was a significant amount of inflammatory cells in the dermis. Nair® cream caused the most severe damage to the skin structure, in which a majority of the defined epidermis layer disappeared, and many inflammatory cells accumulated in the dermis layer. These results show that within 24 h, the two most popular OTC anti-acne medications, BPO and SA, as well as Nair®

hair removal cream induce significant skin toxicity, while LipoLA do not generate any skin reaction within this time frame.

2.8 - Conclusion

In this study, we evaluated the *in vivo* therapeutic potential of liposomal lauric acids (LipoLA) for the treatment of acne infection. The synthesized LipoLA with a size of about 120 nm and a surface zeta potential of -43 mV were prone to fuse with bacterial membranes, thereby directly releasing a high dose of lauric acids into the membranes. Electron microscope images showed that LipoLA caused severe disruption on bacterial membrane structure and morphology including irregularly deformed surface and absent fimbriae, which were correlated with the observed killing of *P. acnes* bacteria. *In vivo* tests further confirmed that LipoLA were able to kill *P. acnes* bacteria through both intradermal injection and topical administration. Finally, a skin toxicity test demonstrated excellent biocompatibility of LipoLA to normal mouse skin. Overall, the results from this work indicate that LipoLA have a high potential to be a new, effective and safe therapeutic agent for the treatment of acne infection.

Chapter 2, in full, is a reprint of the material as it appears in Advanced Healthcare materials, 2013, Dissaya Pornpattananangkul, Victoria Fu, Soracha Thamphiwatana, Li Zhang, Michael Chen, James Vecchio, Weiwei Gao, Che ming Hu, and Liangfang Zhang. The thesis author was the primary investigator and author of this paper.

Chapter 3. Nanoparticle Stabilized Liposomes for Acne Therapy

3.1 - An Introduction to Liposome Stabilization

Despite the numerous advantages that the liposome platform may provide, a major limitation is instability. Because of its 100 nm size and single bilayer structure, liposomes are prone to fuse with one another in order to reduce their surface tension. These resulting fused liposomes are not ideal because the liposome's larger size prevents liposomes from passing through the skin. Additionally, because liposomes have assumed a more stable form, the platform has lost their ability to fuse.

There are a few strategies that have been created to prevent liposome's spontaneous fusion with neighboring liposomes. The most common technique for

overcoming this challenge is coating liposomes with polyethylene glycol (PEG), which not only prevents liposome fusion but enhances in vivo circulation lifetime by suppressing plasma proteins from adsorbing onto the liposome surface. This is advantageous is treating cancerous cells, because these host cells can internalize the entire liposomes by endocytosis. However, this technology is not applicable to bacterial infections due to bacteria-host interaction. Bacteria go through membrane-membrane fusion, and thus PEG coated liposomes are unable to fuse with the bacterial membrane. Thus, it is necessary to redesign liposomes, stabilizing the platform while still retaining the liposomes fusion ability. We report an alternative liposome design, in which liposomes are stabilized by charged nanoparticles in a normal pH environment (pH = 7). However once liposomes reach an acidic environment (pH = 5), charged nanoparticles will detach from the liposome, allowing liposomes the freedom to fuse and deliver drug to bacteria at the target site.[39]

3.2 - Experimental Methods

3.2.1 - *Materials:* Hydrogenated L-a-phosphatidylcholine (Egg PC), 1,2 – di(9Z-octadecenoyl)-3-trimethylammonium propane (DOTAP) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). Doxycycline hyclate, hydroxyethyl cellulose (HEC) and glycerin were obtained from Sigma Aldrich (St Louis, MO). In order to prepare carboxyl-functionalized gold nanoparticles (AuC), the following chemicals were purchased: hydrogen tetrachloroaurate ($HAuCl_4$) and sodium borohydride ($NaBH_4$) were purchased from ACROS Organics, and 3-mercaptopropionic acid (MPA) was purchased from Sigma Aldrich. Brucella broth, Gas-Pak, and agar were purchased from BD (Sparks,

MD). Reinforced clostridial medium was purchase from Oxoid (Hampshire, UK). Institute of Cancer Research (ICR) mice were purchased from Charles River (Wilmington, MA). Polyethylene glycol 400 (PEG 400) was purchased from USB Corporation (Cleveland, OH). Commercial Nair cream, 10% benzoyl peroxide (Clean & Clear®), and 2% salicylic acid (Clean & Clear®) were purchased from a local drug store.

3.2.2 **- *Preparation of carboxyl-terminated gold nanoparticles (AuC):*** AuC nanoparticles were prepared by performing a sodium borohydride redox reaction. An aqueous solution of HAuCl4 (10^{-4} M, 50mL) was reduced by 5 mg of NaBH4 and underwent stirring for one hour to form bare gold nanoparticles. The particles were further functionalized by the addition of MPA (4 x 10^{-4} M) and the solution was allowed to stir overnight. The resulting carboxyl-functionalized gold nanoparticles (AuC) were washed three times and reconcentrated using an Amicon Ultra-4 centrifugal filter with a molecular weight cutoff of 10 kDa (Millipore, Billerica, MA).

3.2.3 **- *Preparation and characterization of AuC-liposomes and AuC-liposome gel:*** Liposomes loaded with doxycline antibiotics were prepared by a vesicle extrusion technique as previously reported. [22] In brief, lipid solution composed of Egg PC and DOTAP (weight ratio = 9:1) was dissolved in chloroform and evaporated under nitrogen gas. The dried lipid film was then rehydrated with a sterile solution of Doxycycline antibiotic dissolved in Milli-Q purified water. The suspension of lipids was vortexed and then sonicated in a bath sonicator (Fisher Scientific FS30D) to produce multilamellar vesicles (MLV). The acquired MLV were further sonicated by a Ti-probe (Branson 450 sonifi er) to produce small unilamellar vesicles (SUV). Lastly, the resulting SUV were

extruded through a 100 nm pore-sized polycarbonate membrane to form doxycycline loaded liposomes. In order to remove free doxycycline drug molecules, liposomes were placed in dialysis cups with a molecular weight cutoff of 10kDa for two days. The UV absorbance of the liposome solution was taken before and after dialysis to establish the doxycycline drug loading percentage. In order to prepare the final form of AuC-stabilized liposomes, the desired molar ratio of AuC was added to the collected liposome solution and was placed under bath sonication for 10 minutes.

The size and zeta potential of the resulting AuC-liposomes were determined using the Malvern Zetasizer ZS (Malvern Instruments, UK), from which the mean diameter of LipoLA was measured through dynamic light scattering (DLS), and the zeta potential through electrophoretic mobility measurements. All characterization measurements were repeated three times at 25 ° C. To prepare AuC-liposomes gel, the mixture of HEC, glycerin, and PEG 400 (7.0, 5.6, and 1.8 wt%) were first swelled by 50 vol% of PBS under stirring for 15 min on a 60 ° C hot plate. The gel was then stirred continuously for 24 h at room temperature. Next, the pre-swelled gel was mixed with 50 vol% of AuC-liposome solution that contained 10 μg/mL of doxycycline and was vortexed until completely homogenized to obtain AuC-liposome gel.

3.2.4 - *Doxycycline Drug Release Study:* 1 mL of bare liposome and AuC liposome solutions were divided into 300 μL volumes and placed in dialysis cups (ThermoScientific) with a molecular weight cutoff of 10kDa. The dialysis cups were placed in distilled water baths with correlating glucose concentrations to account for osmotic pressure. For five days, a sample of both bare liposomes and AuC-liposomes was

measured for UV-absorbance at the doxycycline absorbance wavelength of 345 nm to calculate for doxycycline drug release.

3.2.5 **- *Preparation of P. acnes bacteria:*** (ATCC 6919) (American Type Culture Collection, Manassas, VA) was cultured on a reinforced clostridial medium (RCM) agar plate, under anaerobic condition at 37 ° C for 3 days. A single colony was then inoculated in RCM and cultured at 37 ° C till reaching approximately OD 600 = 1.5 (logarithmic growth phase) under anaerobic condition. Note that for *P. acnes* , OD 600 = 1 is corresponding to a bacterial concentration of 5×10 8 CFU/mL. The bacteria were harvested by centrifugation at 13,000 rpm for 3 min, washed with PBS, repeated 3 times, and then suspended to appropriate amount of PBS for experiment uses.

3.2.6 **- *In vitro antimicrobial efficacy study:*** The antimicrobial activity of AuC-liposomes against *P. acnes* was determined by first, preparing samples of AuC-liposomes at the two desired pH environments of pH = 4 and pH = 7. The pH environments were verified using a pH meter (ThermoScientific). After incubating in the designated pH environments for 10 minutes, the AuC-liposome solutions were diluted to concentrations of (0, 5, 10, 15, 20, 25, 30, and 40 µg/mL). The liposome samples along with a corresponding sample of free doxycycline were incubated with *P. acnes* $(1 \times 10^{7}$ CFU/mL) at 37 ° C under anaerobic condition for 5 h, while *P. acnes* in PBS was used as a negative control. Following incubation, the samples were diluted 1:10 to $1:10^{6}$ in PBS, and 10 µ L of each sample was spotted on RCM agar plates. The samples were incubated at 37 ° C under anaerobic condition for 3 days, and then the CFU of *P. acnes* was quantified.

3.2.7 - **In vivo antimicrobial efficacy study:** To induce *P. acnes* infection, the surface of the mouse ears was first scratched with a 25G needle tip to generate a wound with an area of around 10 mm^2 . Then, 1 μ L of *P. acnes* (1×10^9 CFU/mL) was inoculated onto the wound to yield 1×10^6 CFU of *P. acnes* per ear. After 10 min of inoculation, AuC-liposome gel was applied topically onto the wound. The AuC-liposome gel was applied daily for 2 days successively. After 2 days of drug application, the ears were collected, and the same procedure as described above was performed to quantify the CFU of *P. acnes* on the ears. Commercial Nair cream and PBS gel were used as a positive and a negative control, respectively. Six mouse ears were used for each group (n = 6).

3.2.8 - **Skin toxicity study:** The skin toxicity of AuC-liposomes was tested on the shaved skin of ICR mice. Specifically, the back of the mice was shaved 24 h prior to the study, followed by topically administering AuC-liposome gel gel (1 mg/mL). Blank PBS gel (withoutAuC-liposomes) and empty liposome gel (gel with liposomes that contained no drug) were used as a negative control. Commercial Nair® cream, 10% BPO cream, and 2% salicylic acid (SA) gel were used as positive controls. After 24 h, the skin morphology was examined and imaged. Skin irritation was scored according to Draize's system.[38] For histological observation, the skin was cross-sectioned by an 8 mm biopsy punch. Samples were stained with both hematoxylin and eosin (H&E), and imaged with a microscope. A terminal deoxynucleotidyl transferase duTP nick end labeling (TUNEL) assay was also performed for cell apoptosis. Three mice were used for each group (n = 6) and each assay.

3.2.9 - *Fusion stability study:* To study the long term fusion capacity of liposomes, batches of AuC-liposomes with an EggPC, DOTAP, Rhd-B dye lipid ratio of (8.85: 1: 0.5) were synthesized and placed in 10kDa dialysis units for 48 hours in order to remove free drug. The solution was collected and a desired molar ratio of AuC liposomes was added. The solution was sonicated using a bath sonicator for 10 minutes to ensure AuC nanoparticle attachment to liposomes. Samples were stored and monitored in temperature environments (25°C, 35°C, and 40°C) over a 13 week period. Each week, a sample was taken from storage, and measured for particle size. The sample was then incubated with *P. acnes* for ten minutes. To remove unfused liposomes, the bacteria pellet was collected by centrifugation at 1.3×10^4 rpm and resuspended in water. The fluorescence of the bacteria was measured to study the long term fusion capabilities of this platform.

## 3.3 - Synthesizing Nanoparticle Stabilized Liposomes

A stimuli-responsive, gold nanoparticle-stabilized liposome system, involves the synthesis of positively charged liposomes (diameter = 100 nm) and introducing negatively charged gold nanoparticles (diameter = ~10 nm) that can be absorbed to the liposome surface. Once the gold stabilized liposomes enter an acidic environment (pH = 4), these negatively charged gold nanoparticles become protonated. The gold nanoparticles lose their charge and detach from the liposome's surface, regaining their fusion ability.[39] As a result, this platform design can accomplish two goals. The first accomplishment is creating steric repulsion, preventing fusion with other neighboring liposomes. The second goal is stabilizing liposomes in order to prevent drug leakage. As shown in **Figure 3.1**, gold nanoparticles remain attached to liposomes when placed in a

neutral pH environment (pH = 7.4). However, once these nanoparticle stabilized liposomes are placed in an acidic environment, gold nanoparticles will detach from the liposome surface, allowing liposomes to freely fuse with *P. acnes*.
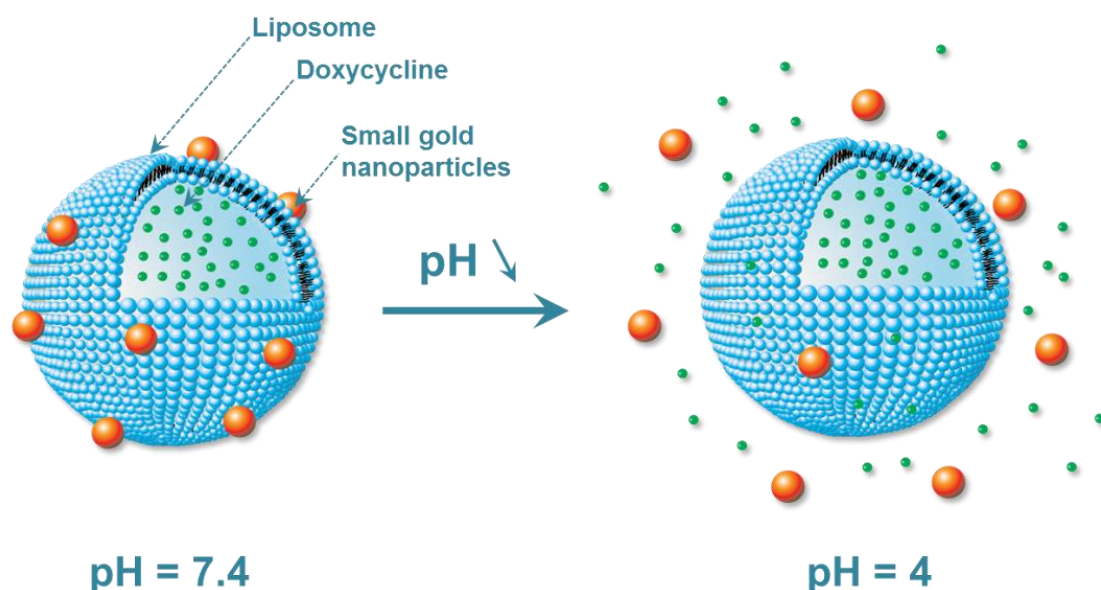

Figure 3.1 - The schematic of the pH responsive liposome platform. In a neutral pH environment (pH = 7.4), AuC absorbs to the liposome's surface, stabilizing the liposome, and prevents inner drug molecules from being released. Once liposomes are placed in acidic environment (pH = 4), AuC particles are no longer negatively charged and detach from the liposome surface, allowing liposomes to regain their fusion capability and release their drug cargo.

Liposomes composed of Egg-PC and DOTA, with weight ratio of (9:1), were loaded with a general tetracycline antibiotic, doxycycline, as a model drug. Doxycycline has a known solubility range of 1-50 mg/mL. In **Figure 3.2**, four samples of various doxycycline concentrations were successfully loaded into liposome. Their particle sizes were measured using dynamic light scattering and were found to have an average particle size of around ~100nm. Doxycycline has an absorbance wavelength of 345 nm and a standard absorbance curve was established for varying concentrations.
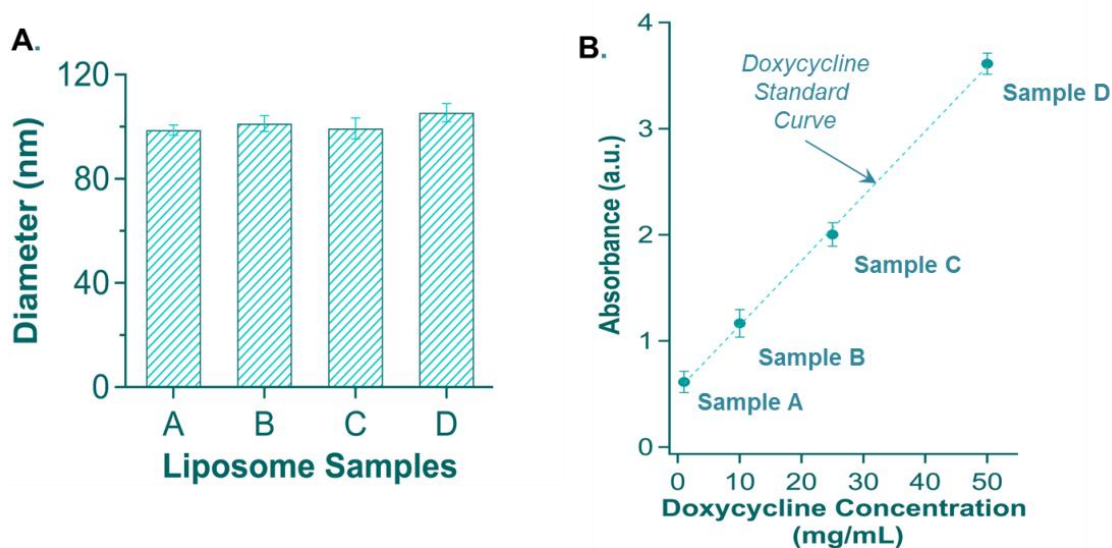
Figure 3.2 - A.) Liposomes were loaded with varying concentration of doxycycline (a) 1 mg/mL, (b) 5 mg/mL (c) 10 mg/mL, and (d) 50 mg/mL and their particle sizes were measured. B.) A standard UV-absorbance curve was found for Doxycycline at an absorbance wavelength of 345 nm.

Carboxyl terminated gold nanoparticles (AuC) were synthesized using a redox reaction of $HAuCl_4$ using $NaBH_4$, and found to have an average diameter of 12 nm. AuC were attached to the surface of doxycycline liposomes, and these nanoparticle stabilized liposomes maintained an average diameter of 110 nm. **Figure 3.3** shows the comparison of size and zeta potential between bare liposomes and functionalized gold nanoparticle stabilized liposomes. Both the change in zeta potential and the slight increase in particle diameter indicates that the absorption of AuC to the liposomes' surface had occurred. In order to confirm the pH response of the nanoparticle stabilized platform, the drug release of AuC-liposomes in varying pH environments was studied for a 5 day time period. As shown in **Figure 3.4**, the doxycycline liposome platforms with AuC attachments were able to remain stable at a normal pH environment (pH = 7.4) and exhibited minimal release.
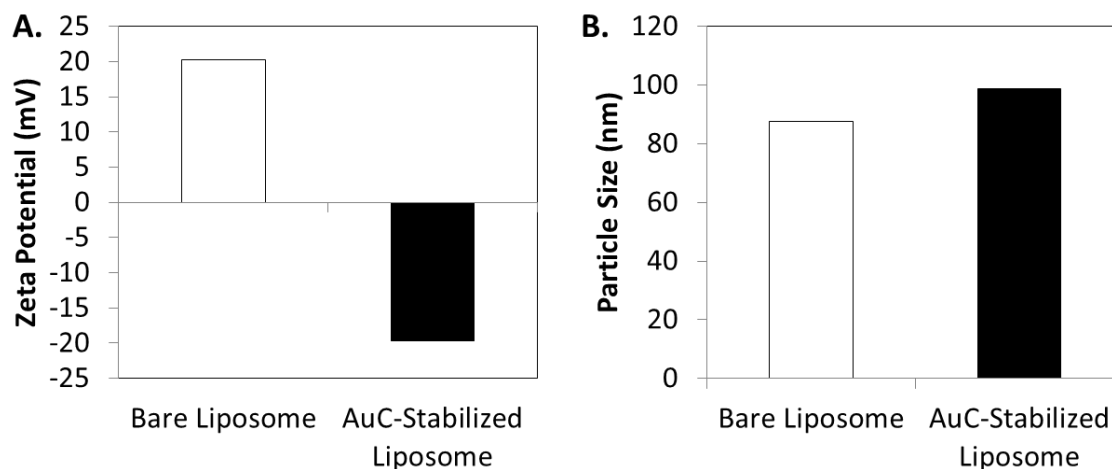
Figure 3.3 - A comparison study of bare liposomes and nanoparticle stabilized liposomes was carried out to measure zeta potential (A) and particle size (B). The change in zeta potential and slight increase in particle size of nanoparticle stabilized liposomes indicates the attachment of AuC to doxycycline loaded liposomes.
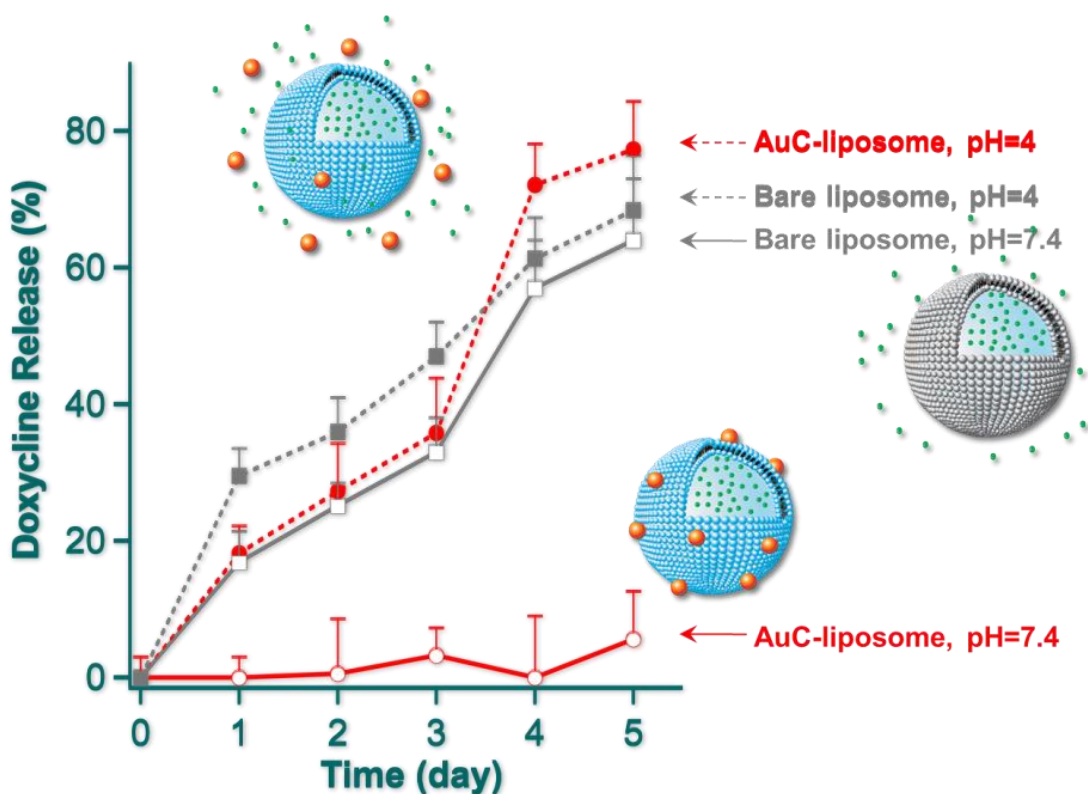

Figure 3.4. - A doxycycline release study based on varying pH environments. Only when AuC-liposomes were placed in acidic environment, did the liposomes exhibit the same drug release activity as bare liposomes. The drug release of bare liposomes showed no difference despite the change in pH environment.

However, AuC-liposomes that were placed in acidic environment showed a 70% drug release over the 5 day time period. Furthermore, this drug release activity followed the same release activity of bare liposomes. As a control, bare liposomes were placed in varying pH environments, but showed no difference in drug release. The significant difference in drug release activity of AuC-liposomes indicates that AuC-liposomes are pH responsive and in a normal pH environment, liposome stabilization was preserved, preventing doxycycline from leaking out of liposomes prematurely.
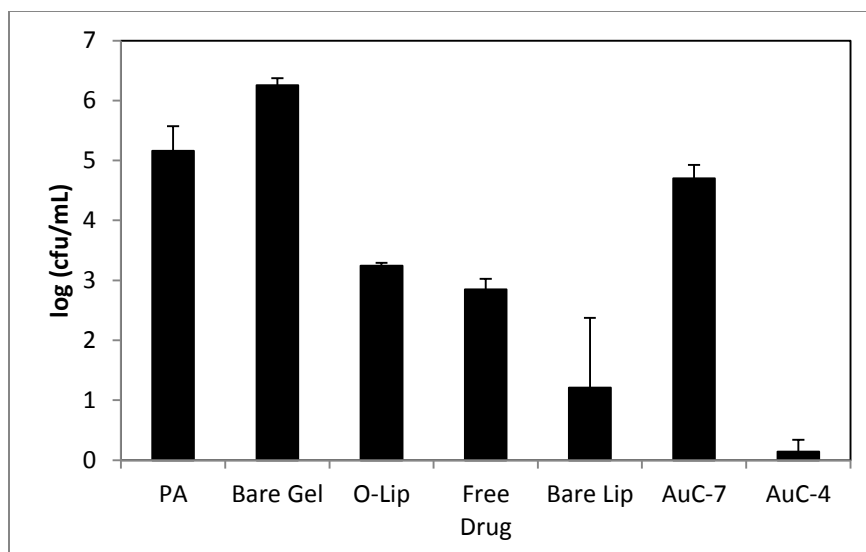
3.4 - The Antimicrobial Efficacy of AuC-Liposomes

In order to study the effectiveness of AuC-liposomes against *P. acnes*, an *in vitro* experiment was conducted where AuC-liposomes were incubated with *P. acnes* (10^7 cfu/mL) for five hours (**Figure 3.5**). AuC-liposomes in a normal pH environment were unable to eradicate *P. acnes* despite the varying concentrations of doxycycline. However, once AuC-liposomes were placed in an acidic environment, AuC-liposomes loaded with a minimal doxycycline concentration of 10μg/mL were able to completely eradicate *P. acnes*. Additionally, 10μg/mL of free doxycycline was unable to show the same antimicrobial activity as AuC-liposomes, and thus *P. acnes* could not be completely eliminated. The improved antimicrobial activity of AuC-liposomes displays the liposome's advantage over free drug in being able to better deliver drug to bacteria by fusing with bacteria membrane and delivering doxycycline into the interior of *P. acnes* causing higher bacteria eradication.
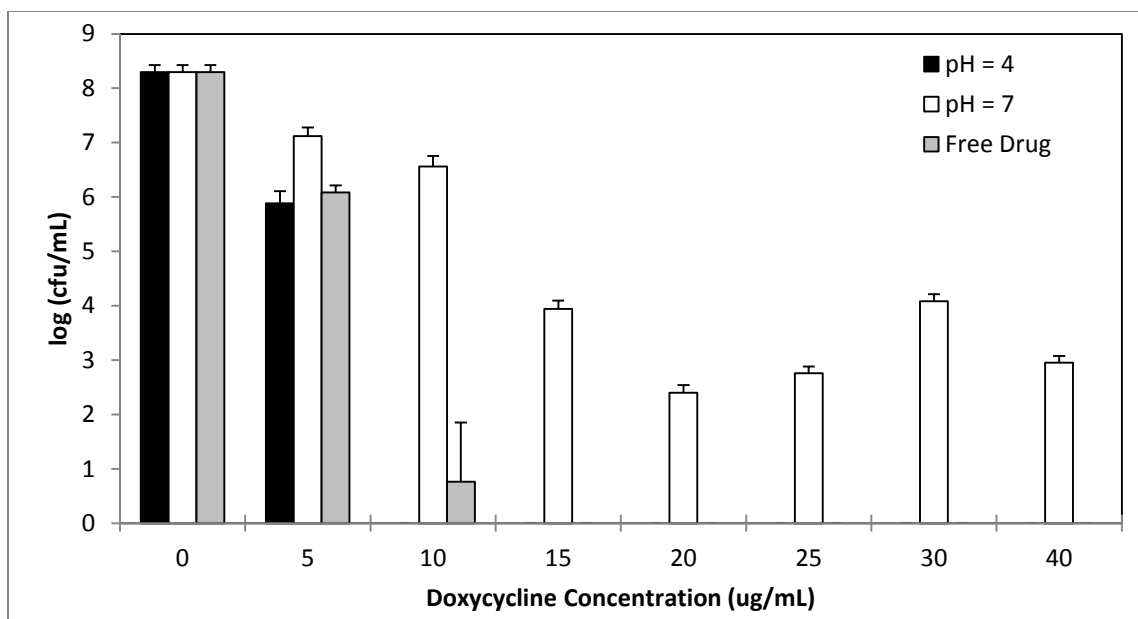
Figure 3.5 - *In vitro Antimicrobial* Efficacy Study of AuC-liposomes. At acidic condition, AuC-liposomes eliminated *P.acnes* within a 5 hour period using a doxycycline concentration of 10 µg/mL. However at a neutral pH environment (pH = 7), AuC-liposomes showed no antimicrobial activity against *P. acnes*. In comparison to AuC-liposomes, a corresponding concentration of free doxycycline (10 µg/mL) was unable to completely eradicate bacteria.

For verification, an *in vivo* antimicrobial efficacy was also conducted to study the antimicrobial activity of AuC-liposomes in a live animal model. Since mice are unable to generate *P. acnes*, a wound ear model was used to imbed *P. acnes* within the ear, and an AuC-liposome gel formulation was used to treat the *P. acnes* site for a sample size n = 6. Bare gel, empty liposomes, free drug, and bare liposomes were used as control samples. After a 48 hour treatment period, AuC-liposome gel placed in an acidic environment was found to have the highest antimicrobial activity in comparison to free drug and bare liposome samples loaded with the same amount of doxycycline (10 µg/mL).
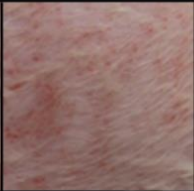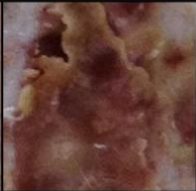
Figure 3.6 - *In vivo* antimicrobial Efficacy Study of AuC-liposomes. The topical treatment of AuC-liposomes at acidic condition showed the highest antimicrobial activity against P. acnes, while at pH = 7.4, AuC-liposomes showed no significant antimicrobial activity. Control samples of (only *P. acne*, bare HEC gel, empty liposomes, free doxycycline, and bare liposomes loaded with doxycycline) were used. Empty liposomes, free drug, and bare liposome control samples showed some antimicrobial activity against *P. acnes.*

However AuC-liposomes that remained in a normal pH environment had no effect on *P. acnes* (**Figure 3.6**). The empty liposome control samples were found to still have some antimicrobial activity due to the liposomes ability to fuse with *P. acnes* and disrupt the bacteria membrane.

3.5 - A Toxicity Study of AuC-Liposomes

AuC-liposomes were tested for any induced toxicity by topically applying the gel formulation on the shaven epidermis of mice for 7 days. Using the Draize scoring for irritation, AuC-liposomes were found to have developed no erythema or edema irritation. The two most popular over-the-counter drugs for acne therapy were also tested for toxicity and both showed moderate erythema in the epidermis.[38] Furthermore, nair was

used as a positive control and generated severe erythema with eschar formation and severe edema indicating in depth irritation.

	Normal	AuC-liposome	Salicylic Acid	BPO	Nair
Morphology					
Erythema	0	0	2	2	4
Edema	0	0	0	0	3

Figure 3.7 - A seven day, *in vivo* toxicity study of the AuC-lipsoome topical gel formulation was conducted and scored using the Draize scoring system for irritation. AuC-liposomes were found to generate no apparent erythema or edema. Both salicylic acid, and benzoyl peroxide samples generated moderate erythema. Nair was used as a positive control and exhibited extreme erythema and edema.

	Normal	AuC-Liposome	Salicylic Acid	BPO	Nair
H&E					
TUNEL					
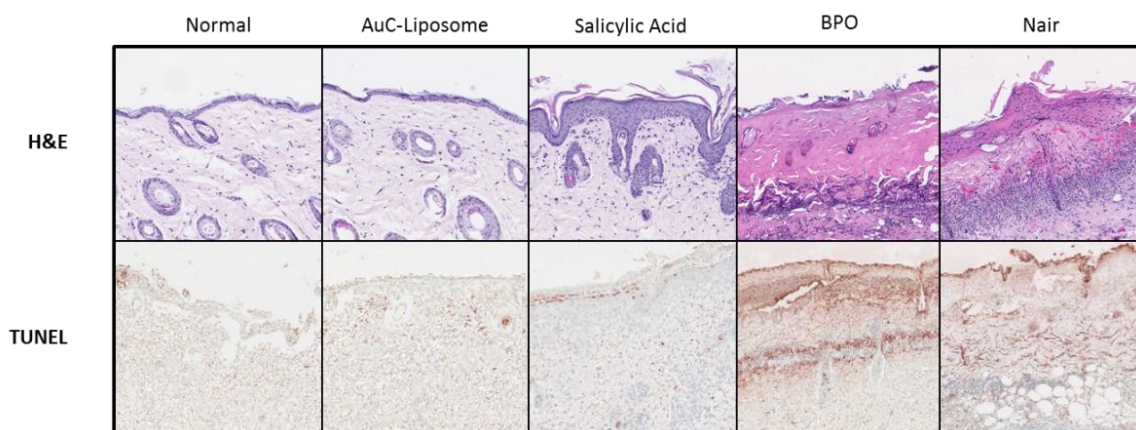
Figure 3.8 - An in vivo toxicity study comparing AuC-liposomes to the top two over the counter drugs available for acne therapy. The AuC-liposome formulation generated no inflammation and no significant cell death. Salicylic acid showed moderate inflammation, causing the detachment of the corneum stratum and thickening of the epidermis. Both BPO and Nair generated severe inflammation with major accumulation of lymphocytes within the dermis layer, degradation of the stratum corneum, and necrotic cells.

For further analysis, H&E staining and TUNEL assays were also performed to measure irritation and cell apoptosis. H&E nuclear staining showed no induced inflammation in tissue that had been treated with AuC-liposomes. However, in tissue treated with salicylic acid, the stratum corneum is detached, and the epidermis layer has thickened. The stratum corneum is the outermost layer of the epidermis that consists of dead cells. Keeping the integrity of this outermost layer is important because it forms a barrier to protect the underlying tissue. In both the benzoyl peroxide and Nair treated tissue, the stratum corneum had been eroded and there was a significant increase in the density of lymphocytes that had accumulated within the dermis layer, showing that inflammation had progressed into the dermis layer. A TUNEL assay was performed in order to assess the amount of cells with severe DNA damage and necrotic cells generated in the skin tissue. In **Figure 3.8**, both AuC-liposomes and salicylic acid samples showed the same, minimal cell death as normal tissue. However in BPO and Nair, necrosis had developed in both the epidermis and dermis layers of the tissue.

3.6 - The Long Term Stability of AuC-Liposomes

In order to monitor the stability of AuC stabilized-Doxy Liposomes over a 3 month period, the nanoplatform's fusion capacity with *P. acnes* was measured weekly. High temperature testing is commonly used to dramatically alter the nature of the interfacial film and induce fusion. Thus, large batches of AuC-liposomes were stored at temperatures of 25°C, 35°C, and 40°C for a 3 month period and the particle size and fusion activity were measured (**Figure 3.9**). Using dynamic light scattering, AuC-liposomes stored at room temperature showed minimal increase in particle size. The

fusion capacity of the liposome formulation was also monitored at these three designated temperatures, in order to study how long particles could retain the AuC attachment and stabilize liposomes.
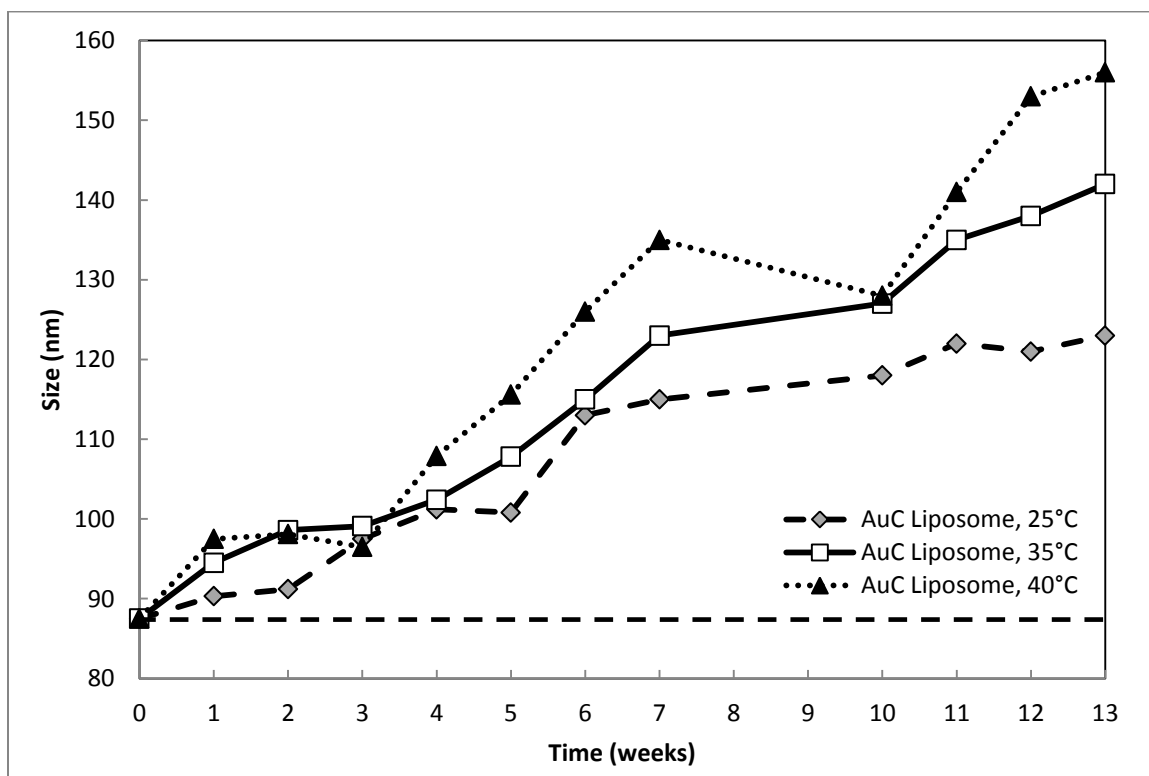
Figure 3.9 - Particle size of AuC-liposomes were monitored for 3 months at temperatures of 25°C, 35°C, and 40°C. At room temperature, liposomes showed minor increase in particle diameter from 87.7 nm to 119.5 nm. The diameter of AuC-liposomes stored at higher temperatures showed a greater increase from 87.7 nm to 157.6 nm.

At room temperature, liposomes showed a minor increase over 3 months from 87.7 to 119.5 nm. Liposomes stored at 35°C and 40°C, were found to have a greater increase in particle size. At the end of the 3 month period, the diameter of liposomes had increase from 87.7 to 157.6 nm. At these two higher temperatures, the fluorescence intensity of these liposomes were significantly higher, which indicated that fusion of liposomes with bacteria was occurring despite being placed in a pH environment of 7.4.

This signifies that AuC was no longer attached to the liposome surface and liposomes were free to fuse with bacteria. In comparison, liposomes stored at room temperature were found to maintain stability and displayed minimal fusion with bacteria over a 3 month period.
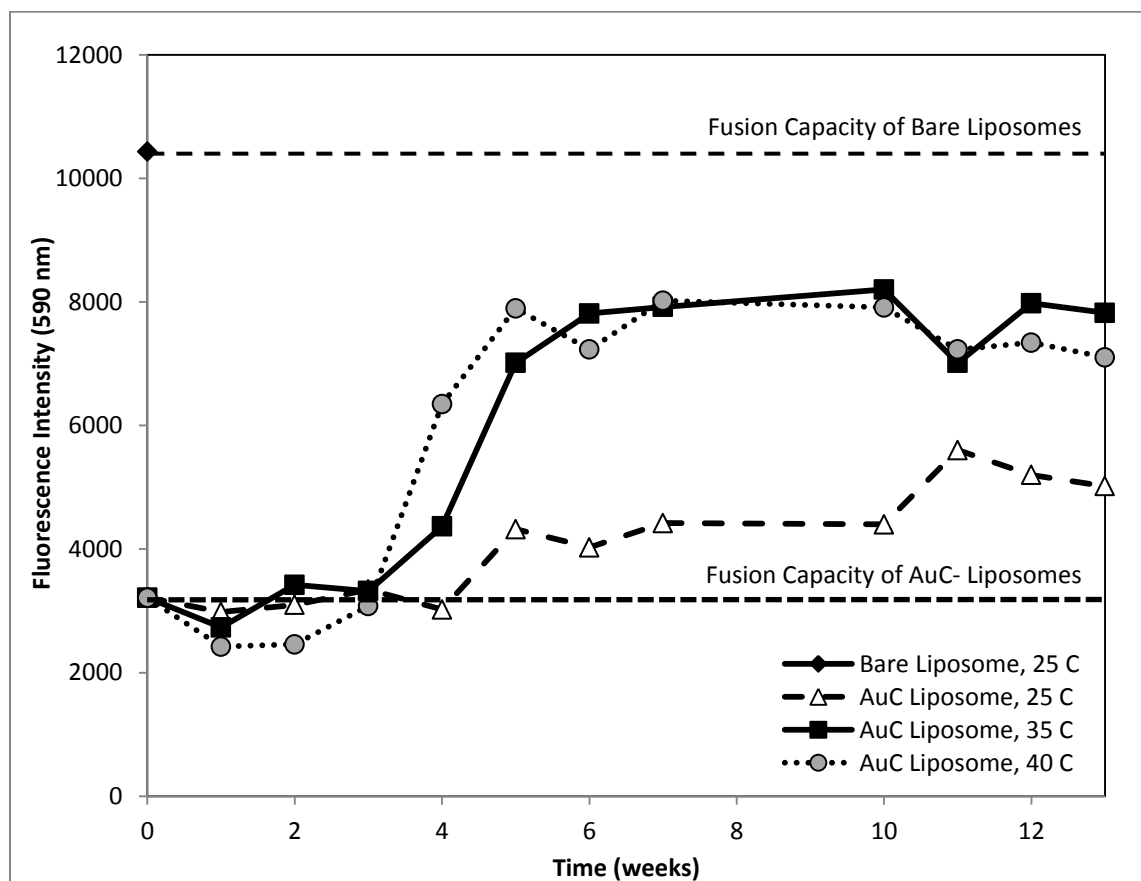
Figure 3.10 - The fusion capability of liposomes was examined over a 3 month period in order to evaluate the long-term stability of AuC-liposomes. When liposomes were stored at 25°C, there was minimal fusion between liposomes and P. acnes. In contrast, liposomes that were stored at higher temperatures of 35°C and 40°C readily fused with bacteria, generating higher fluorescence intensity.

Fusion Capacity at Week 6
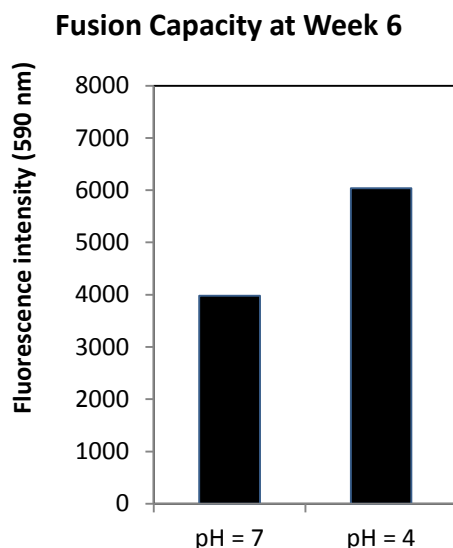
Figure 3.11 - A fusion capacity comparison study was performed on stored AuC-liposomes before and after a pH environment adjustment from pH = 7.4 to pH = 4. A higher fluorescent intensity was detected in bacteria after being incubated with AuC-liposomes in an acidic environment (pH = 4). This indicates that AuC detached from liposomes and liposomes were able to fuse with P. acnes bacteria.

It is important to note that several additional observations were made over a 3 month period. At week 6, a sample of AuC-Doxy Liposomes stored at room temperature was taken and its pH was adjusted to an acidic environment (pH = 4). After the pH adjustment, liposomes showed enhanced fusion with P. acnes, showing the retention of the AuC-liposome's fusion capacity (**Figure 3.11**). In addition, the polydispersity index of liposomes kept at a heated temperature increased significantly over time from 0.197 to 0.348. This indicates that liposomes no longer held a uniform size distribution and fusion had occurred between liposomes. Furthermore, by the third week of storage, the coloring of doxycycline had significantly changed. This color change observation is a direct correlation with the stability of the antibiotic and is not tied to the stability of the liposome platform.

3.7 - Conclusion

In this study, the stability, antimicrobial efficacy, toxicity, and drug release of AuC-liposomeswe were evaluated as a potential acne treatment. AuC-liposomes were found to have a particle size of 110nm with a zeta potential of -20 mV. AuC-liposomes only exhibited a drug release response when placed in an acidic environment of pH = 4. Both *in vitro* and *in vivo* antimicrobial studies showed that upon placing AuC-liposomes in an acidic environment loaded with a 10 μg/mL concentration of doxycycline, liposomes were able to significantly eradicate *P. acnes*. In addition, a skin toxicity study demonstrated that AuC-liposomes were highly biocompatible and generated no inflammation or cell apoptosis. Finally, this liposome platform stored at room temperature showed high stability with minimal increase of particle size and the retention the liposome's fusion capability. As a result, AuC-liposomes are a promising platform for the topical treatment of P. acnes because the platform is both safe and effective in treating acne infection.

Chapter 3, in full, uses the technology of US Provisional Patent PCT/US2011/028014 (2011)¸ Dissaya Pornpattananangkul and Liangfang Zhang, and is a future manuscript submission to ACS Nano, 2014, Victoria Fu, Soracha Thamphiwatana, Weiwei Gao, and Liangfang Zhang. The thesis author was the primary investigator of this manuscript.

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