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A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy

in

Bioengineering

by

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2012
The Dissertation of Dissaya Pornpattananangkul is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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Co-Chair

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University of California, San Diego

2012
DEDICATION

This dissertation is dedicated to my beloved family, The Pornpattananangkuls and the Tovikkais. This work has been possible because of their unconditional love and never-ending support.
EPIGRAPH

“I never did a day's work in my life. It was all fun.”

*Thomas A. Edison*
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Chapter 2, in full, is a reprint of the material as it appears in ACS Nano, 2010, Dissaya Pornpattananangkul, Sage Olson, Santosh Aryal, Marta Sartor, Chun-Ming Huang, Kenneth Vecchio, and Liangfang Zhang, and, in full, in Journal of the American Chemical Society, 2011, Dissaya Pornpattananangkul, Li Zhang, Sage Olson, Santosh Aryal, Marygorret Obonyo, Kenneth Vecchio, Chun-Ming Huang, and Liangfang Zhang. The dissertation author was the primary investigator and author of these papers.

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ABSTRACT OF THE DISSERTATION

Antimicrobial Nanoparticle for the Treatment of Bacterial Infection

by

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Doctor of Philosophy

University of California, San Diego, 2012

Professor Michael J. Heller, Chair
Professor Liangfang Zhang, Co-Chair

Liposomes are spherical lipid vesicles with bilayered membrane structure, which have been recognized as one of the most widely used carriers for delivering a myriad of pharmaceuticals. Liposomes can carry both hydrophilic and hydrophobic agents with high efficiency and protect them from undesired effects of external conditions. However, the applications of liposomes are usually limited by their instability during storage. They are inclined to fuse with one another immediately after preparation, resulting in undesired mixing, increase in size, and payload loss. To
overcome this limitation, this dissertation will focus on the technology to stabilize liposomes during storage and destabilize at specific conditions in order to allow controllable therapeutic release, as well as demonstrate their application to treat one of the bacterial infection diseases, acne vulgaris.

The first area of this research is stimuli-responsive liposomes development, where the liposomes are stabilized by introducing gold nanoparticles to adsorb to their surface. As a result, the liposomes are prevented from fusing with one another and undesirable payload release during storage or physiological environments. Moreover, therapeutic is controllably released depending on environment conditions, such as acidic pH and bacterial virulence factor. In case of acid-responsive liposomes, the bound gold nanoparticles can effectively prevent liposomes from fusing with one another at neutral pH value, while at acidic environment (e.g. pH<5), the gold particle stabilizers will fall off from the liposomes, thereby reinstalling the fusion activity of liposomes. The fusion activity of the stabilized liposomes is found to be 25% at pH=7, in contrast to 80% at pH=4. Another stimulus that can activate drug release from liposomes is virulence factor released from bacteria themselves, such as bacterial toxin. When nanoparticle-stabilized liposomes encounter with bacteria that secrete toxin, the toxin will insert into the liposome membranes and form pores, through which the encapsulated therapeutic agents are released. The released drugs subsequently impose antimicrobial effects on the toxin-secreting bacteria. It was observed that in the presence of toxin-secreting bacteria, 100% of the encapsulated
antibiotics were released from the gold nanoparticle-stabilized liposomes and bacterial growth was effectively inhibited by the released antibiotics in 24 h.

The second area is to demonstrate an application of the invented technology to treat acne vulgaris by delivering therapeutics to the acne-causing bacteria, named *Propionibacterium acnes* (*P.acnes*). First, lauric acid (LA), an antimicrobial with strong activity against *P. acnes*, is encapsulated in liposomes (LipoLA), which is shown to effectively kill the bacteria by fusion with the bacterial membrane, resulting in a direct insertion of LA molecules to the membrane and destruction of its surface structure *in vitro* and *in vivo*. The system is then further improved by the acid-responsive technology based on the fact that the acne lesions on human skin are typically acidic. Demonstrated by fluorescent and antimicrobial experiments, the bound gold nanoparticles effectively prevent LipoLA from fusing with one another at neutral pH value. However, at acidic condition, the gold particles detach from LipoLA surface, allowing the fusion with *P.acnes* membrane and lauric acid delivery, resulting in a complete killing effect.

The stimuli-responsive liposomes presented here provide a new, safe, and effective approach to treat bacterial infections. They can be broadly applied to treat a variety of infections caused by bacteria that reside in acidic environment and secrete pore-forming toxins.
Chapter 1 Introduction

1.1 Infection Disease and Antimicrobial Therapeutic

Upon invasion of the epithelial surfaces, infectious microorganisms spread throughout the body via the circulatory system. They are then removed from the blood by macrophages which are present in all major organs such as liver, spleen and bone marrow[1]. After being phagocytosed by macrophages, the infectious microorganisms are trapped in phagosomes, which then fuse with lysosomal granules inside cell cytoplasm leading to phagolysosomes. Subsequently, oxygen-dependent or oxygen-independent bacterial killing mechanisms induced by enzymes inside the phagolysosomes occur to digest the trapped microorganisms. However, many
microorganisms are able to evade the macrophage digestion via escaping from the phagosomes, inhibiting the phagosome-lysosome fusion, withstanding the lysosomal enzymes, or resisting oxidative and non-oxidative killing mechanisms. These bacterial defense mechanisms make intracellular infections difficult to eradicate resulting in infectious diseases that range from staph infections to tuberculosis [1].

An antimicrobial refers to a substance that kills or inhibits the growth of microorganisms. Since the discovery of antimicrobial drugs in the 1960s[2], many infectious diseases have been overcome. Typically, antimicrobials kill bacteria by binding to some vital compounds of bacterial metabolism, thereby inhibiting the synthesis of functional biomolecules or impeding normal cellular activities. For instance, β-lactams such as penicillins and cephalosporins inhibit bacteria cell wall synthesis; tetracyclines, macrolides, and clindamycin inhibit protein synthesis; metronidazole and quinolones inhibit nucleic acid synthesis; and sulphonamides and trimethoprim have an inhibitory effect on enzyme synthesis. Some antimicrobials such as penicillin are only effective against a narrow range of bacteria, whereas others, like ampicillin, kill a broad spectrum of Gram-positive and Gram-negative bacteria[3]. Despite the great progress in antimicrobial development, many infectious diseases, especially intracellular infections, remain difficult to treat. One major reason is that many antimicrobials are difficult to transport through cell membranes and have low activity inside the cells, thereby imposing negligible inhibitory or bactericidal effects on the intracellular bacteria. In addition, antimicrobial toxicity to healthy tissues poses a significant limitation to their use. Aminoglycosides, for instance,
causes ototoxicity and nephrotoxicity and have to be given in controlled dosages. Another major issue with antimicrobials stems from the acquired resistance of infectious microbes. In 2002, more than 70% of bacteria causing hospital-acquired infections were resistant to at least one common antimicrobial in the United States. To address these issues, alternative antimicrobial drug delivery strategies have been proposed[1].

1.2 Nanoparticles for Antimicrobial Drug Delivery

Over the last few decades, the applications of nanotechnology in medicine have been extensively explored in many medical areas, especially in drug delivery. Nanotechnology concerns the understanding and control of matters in the 1-100 nm range, at which scale materials have unique physicochemical properties including ultra small size, large surface to mass ratio, high reactivity and unique interactions with biological systems[4]. By loading drugs into nanoparticles through physical encapsulation, adsorption, or chemical conjugation, the pharmacokinetics and therapeutic index of the drugs can be significantly improved in contrast to the free drug counterparts. Many advantages of nanoparticle-based drug delivery have been recognized, including improving serum solubility of the drugs, prolonging the systemic circulation lifetime, releasing drugs at a sustained and controlled manner, preferentially delivering drugs to the tissues and cells of interest, and concurrently delivering multiple therapeutic agents to the same cells for combination therapy [4-6].
Moreover, drug-loaded nanoparticles can enter host cells through endocytosis and then release drug payloads to treat microbes-induced intracellular infections. As a result, a number of nanoparticle-based drug delivery systems have been approved for clinical uses to treat a variety of diseases and many other therapeutic nanoparticle formulations are currently under various stages of clinical tests [4, 7]. Knowing the vast scope of nanoparticle drug delivery, here we will only focus on the development and application of nanoparticles for antimicrobial drug delivery through various mechanisms as illustrated in Figure 1.1.

![Figure 1.1. Mechanism of antimicrobial drug delivery to microorganism. (1) nanoparticle fuses with microbial cell wall and releases drug inside the cell; (2) nanoparticle binds to cell wall and releases drug molecules, following with drug fusion through cell wall.](image-url)
As shown in Figure 1.2, a few types of nanoparticles including liposomes, polymeric nanoparticles, solid lipid nanoparticles and dendrimers have been widely investigated as antimicrobial drug delivery platforms, of which several products have been introduced into pharmaceutical market. This chapter will provide the current status, mechanisms of action, and structure activity relationship of these nanoparticle-based antimicrobial delivery systems.

Figure 1.2. Schematic illustration of four nanoparticle platforms for antimicrobial drug delivery: (a) liposome, (b) polymeric nanoparticle, (c) solid lipid nanoparticle, and (d) dendrimer. Black circles represent hydrophobic drugs; black squares represent hydrophilic drugs; and black triangles represent either hydrophobic or hydrophilic drugs.
1.2.1 Liposomes

Liposomes are spherical lipid vesicles with a bilayered membrane structure consisting of amphiphilic lipid molecules [8]. Liposome structure was first described in 1965[9], and they were proposed as a drug delivery nanoparticle platform in 1970s[10]. After extensive studies on their fundamental properties including lipid polymorphisms, lipid-protein and lipid-drug interactions, and mechanisms of liposome disposition in 1980s, the application potential of liposomes as a drug delivery vehicle was thoroughly recognized and started being transferred to practice. Liposomes were initially introduced to the cosmetic market by Dior in 1986. In 1995, Doxil (doxorubicin liposomes) became the first liposomal delivery system approved by the Food and Drug Administration (FDA) to treat AIDS associated Kaposi’s sarcoma [11, 12]. Liposomal drug delivery system can be made of either natural or synthetic lipids. One of the most commonly used lipids in liposome preparation is phosphatidylcholine, which is an electrically neutral phospholipid that contains fatty acyl chains of varying degrees of saturation and length. Cholesterol is normally incorporated into the formulation to adjust membrane rigidity and stability. Structurally, liposomes can be classified into multilamellar vesicles (MLVs), which consist of multiple phospholipid bilayer membranes, and unilamellar vesicles (ULVs), which have a single lipid bilayer. ULVs can be further classified into small unilamellar vesicles (SUVs) and large unilamellar vesicles (LUVs) depending on their size range[13]. Methods for preparing liposomes can take into consideration parameters such as the physicochemical characteristics of the liposomal ingredients,
materials to be contained within the liposomes, particle size, polydispersity, surface
zeta potential, shelf-time, batch-to-batch reproducibility, and the possibility for large-
scale production of safe and efficient products. Liposomes, ULVs in particular, do not
form spontaneously. Rather, liposomes form when a sufficient amount of energy
(e.g., via sonication, homogenization, shaking, or heating) is supplied to
phospholipids placed in water. Typical methods for generating liposomes include
sonication method[14], (e.g., low shear rates can result in MLVs and high shear rates
can generate ULVs), extrusion method, and heading method [15].

Currently, liposomes are the most widely used antimicrobial drug delivery
system. One of the distinguishing features of liposomes is its lipid bilayer structure,
which mimics cell membranes and can readily fuse with infectious microbes. By
directly fusing with bacterial membranes, the drug payloads of liposomes can be
released to the cell membranes or the interior of the bacteria. The unique structure of
liposomes, a lipid membrane surrounding an aqueous cavity, enables them to carry
both hydrophobic and hydrophilic compounds without chemical modification. On the
other hand, by attaching targeting ligands such as antibody, antibody segments,
aptamer, peptides and small molecule ligands to the surface of the liposomes, they
can selectively bind to microorganisms or infected cells and then release the drug
payloads to kill or inhibit the growth of the microorganisms.

AmBisome (NeXstar Pharmaceuticals, San Dimas, USA) is an FDA approved
liposomal formulation of amphotericin B (AMB), which has been widely used in the
clinic to treat Candida spp, Aspergillus spp, Fusarium spp, and other fungi infections
in neutropenic, visceral leishmaniasis, and methylmalonic acidaemia patients [16-18]. In the AmBisome formulation, AMB are intercalated into the phospholipid bilayer of liposomes consisting of hydrogenated soy phosphatidylcholine, cholesterol, and distearoyl phosphatidylglycerol (DSPG) [19]. Freeze-fracture electron microscopy results have shown that AmBisome delivers its drug content through an absorption mechanism. For example, AmBisome attached to the outer cell wall of *Candida glabrata* [20] and release AMB to disrupt fungal membranes[21, 22]. Adler-Moore J. et al. have observed great specificity of AmBisome to the site of fungal infections. Following the injection of fluorescently-labeled AmBisome into *Candida*-infected mice, the localization of fluorescent liposomes has been observed at the sites of fungal infections [23]. Because of its liposomal structure, AmBisome has shown greater pharmacokinetics than free AMB drug, including prolonged systemic circulation half-life, reduced plasma clearance rate, decreased renal toxicity, and most importantly, enhanced therapeutic efficacy [24].

Polymyxin B-loaded loposome represents another successful example of liposomal antimicrobial drug delivery. Polymyxin B has been recognized for treating *P. aeruginosa* related infections (e.g., pneumonias and chronic bronchopneumonia’s of cystic fibrosis). However, its systemic use has been limited due to toxic side effects such as nephrotoxicity, ototoxicity and neuromuscular blockade. It has been reported that liposomal encapsulation of polymyxin B dramatically diminished the drug’s side effects and improved its antimicrobial activity against resistant strains of *P. aeruginosa* [25]. The action mechanism of liposomal polymyxin B against *P.
*P. aeruginosa* has been recognized as membrane fusion. Transmission electron microscopy (TEM), flow cytometry and fluorescent resonance energy transfer studies have revealed lipid reorganizations in *P. aeruginosa* membranes upon incubation with polymyxin B-loaded liposomes [26]. Membrane fusion between liposomes and bacteria is a rapid and spontaneous process driven by non-covalent forces such as van der Waals force and hydrophobic interactions that minimize the system’s free energy. Antibiotic efflux is a widely accepted mechanism of microbial drug resistance, in which proteinaceous transports located in bacterial membranes preferentially pump antimicrobial drugs out of the cells [27]. When liposomes fuse with cell membranes, a high dosage of drug contents is immediately delivered to the bacteria, which can potentially suppress the antimicrobial resistance of the bacteria by overwhelming the efflux pumps, thereby improving drug’s antimicrobial activity.

Many other liposome-based antimicrobial drug delivery systems have also been developed for various applications [27]. Ampicillin-loaded liposomes have shown elevated drug stability and higher antimicrobial activity than free drug against *Salmonella typhimurium* [28, 29]. Benzyl penicillin-loaded liposomes have shown complete growth inhibition of penicillin-sensitive strain of *Staphylococcus aureus* at lower drug concentration and shorter exposure time than free benzyl penicillin [30]. Liposomal ciprofloxacin, a fluoroquinolone antibiotic, has effectively inhibited the number of *Salmonella dublin* in mouse spleen [31]. Liposomal gentamicin and streptomycin, which belong to aminoglycoside antibiotics, have successfully treated mice and guinea pigs infected with *Brucella* spp.[32]. It has also been reported that
liposomal vancomycin and teicoplanin have significantly enhanced intracellular killing of methicillin-resistant *Staphylococcus aureus* (MRSA) [33]. Table 1.1 summarizes other antimicrobial liposomes, many of which have been in clinical use for years.

**Table 1.1.** Liposomes for antimicrobial drug delivery

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Drug</th>
<th>Targeted Microorganism</th>
<th>Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>hydrogenated soy phosphatidylcholine, cholesterol, and distearoylphosphatidylglycerol (DSPG)</td>
<td>amphotericin B</td>
<td><em>Aspergillus fumigatus</em></td>
<td>targeted drug delivery at infection site [34]</td>
</tr>
<tr>
<td>1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) and cholesterol</td>
<td>polymyxin B</td>
<td><em>Pseudomonas aeruginosa</em></td>
<td>1) decreased bacteria count in lung 2) increased bioavailability 3) decreased lung injury caused by bacteria [35]</td>
</tr>
<tr>
<td>soybean phosphatidylcholine (PC) and cholesterol</td>
<td>ampicillin</td>
<td><em>Micrococcus Luteus</em> and <em>Salmonella typhimurium</em></td>
<td>1) increased stability 2) full biological activity of Ampicillin was observed [28]</td>
</tr>
<tr>
<td>dipalmitoylphosphatidylcholine, dipalmitoyl-phosphatidylglycerol, and cholesterol</td>
<td>ciprofloxacin</td>
<td><em>Salmonella dublin</em></td>
<td>1) decreased mortality of animals 2) distribution of liposomes to all areas of infection [31]</td>
</tr>
<tr>
<td>dipalmitoylphosphatidylcholine (DPPC), cholesterol, and dimethylammonium ethane carbamoyl cholesterol</td>
<td>benzyl penicillin</td>
<td><em>Staphylococcus aureus</em></td>
<td>lower drug concentrations and shorter time of exposure [30]</td>
</tr>
<tr>
<td><strong>Formulation</strong></td>
<td><strong>Drug</strong></td>
<td><strong>Targeted Microorganism</strong></td>
<td><strong>Activity</strong></td>
</tr>
<tr>
<td>-----------------</td>
<td>----------</td>
<td>---------------------------</td>
<td>--------------</td>
</tr>
</tbody>
</table>
| phosphatidylcholine, cholesterol, and phosphatidylinositol | netilmicin | *Bacillus subtilis* and *Escherichia coli* | 1) reduction in toxicity  
2) increased circulation half-life  
3) increased survival rate of animal model | [36] |
| partially hydrogenated egg phosphatidylcholine (PHEPC), cholesterol, and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-(polyethylene glycol-2000) (PEGDSPE) | gentamicin | *Klebsiella pneumoniae* | 1) increased survival rate of animal model  
2) increased therapeutic efficacy | [37] |
| phosphatidyl glycerol, phosphatidyl choline, and cholesterol | streptomycin | *Mycobacterium avium* | increased antimicrobial activity | [38] |
| hydrogenated soy phosphatidylcholine, cholesterol, and distearoylphosphatidylglycerol (DSPG) | amikacin | gram-negative bacteria | prolonged drug and exposure | [39] |
| stearylamine (SA) and dicetyl phosphate | zidovudine | Human immunodeficiency virus | enhanced targeting of ZDV to lymphatics | [40] |
| egg phosphatidylcholine, diacetylphosphate, and cholesterol | vancomycin or teicoplanin | methicillin-resistant *Staphylococcus aureus* (MRSA) | 1) enhanced each drug uptake by macrophages  
2) enhanced intracellular antimicrobial effect of each drug | [33] |
1.2.2 Other Nanoparticle Platforms

Even though liposomes are the most widely used antimicrobial drug delivery system because of their various advantages as mentioned in the previous section, other nanoparticle platforms have also been investigated extensively. These nanoparticles include polymeric nanoparticles, solid lipid nanoparticles (SLNs), and dendrimers.

Biocompatible and biodegradable polymers have been used extensively in the clinic for controlled drug release. The annual worldwide market of polymer-based controlled release systems is about $60 billion and they are given to over 100 million patients each year [41]. The first polymer-based drug delivery system was developed by Langer and Folkman in 1976 for macromolecule delivery[42]. However, the initial polymeric nanoparticles possessed poor therapeutic efficacy because of their rapid clearance by the reticuloendothelial system (RES) after intravenous administration. This limitation was overcome after the discovery of long-circulating stealth polymeric nanoparticles in 1994 [43]. Polymeric nanoparticles possess several unique characteristics for antimicrobial drug delivery; they are structurally stable and can be synthesized with a sharper size distribution; particle properties such as size, zeta potentials, and drug release profiles can be precisely tuned by selecting different polymer lengths, surfactants, and organic solvents during the synthesis; and the surface of polymeric nanoparticles typically contain functional groups that can be chemically modified with either drug moieties or targeting ligands.
Solid lipid nanoparticles (SLNs) are another antimicrobial drug delivery platform that has attracted much attention since 1990s. SLNs are mainly comprised of lipids that are in solid phase at the room temperature and surfactants for emulsification. The typical methods of preparing SLNs include spray drying [44], high shear mixing, ultra-sonication [45], and high pressure homogenization (HPH) [46]. Several unique properties of SLNs make them a promising antimicrobial drug delivery platform, leading to a few cosmetics and pharmaceutical products for skin care applications. Firstly, SLNs contain occlusive excipients that, upon application on skin, readily form a thin film to reduce water evaporation and retain skin moisture. Secondly, SLNs are stable in water and dermal cream and therefore can be readily incorporated into cosmetic and skin care products[47]. Lastly, simple manufacturing techniques such as high pressure homogenization make it possible to produce SLNs in a large-scale and reproducible manner. Moreover, the preparation of SLNs does not require any organic solvents, which could be difficult to remove after nanoparticle synthesis.

Dendrimers are defined as highly ordered and regularly branched globular macromolecules produced by stepwise iterative approaches. The structure of dendrimers consists of three distinct architectural regions: a focal moiety or core, layers of branched repeat units emerging from the core, and functional end groups on the outer layer of repeat units [48]. In 1978, the first iterative cascade synthetic procedure for branched amines was discovered by Vögtle et al.[49]. A few years later, highly branched l-lysine-based dendrimers were patented[50]. In 1984, Tomalia et al.
reported the synthesis and characterization of the first family of polyamidoamine (PAMAM) dendrimers, which has become the most popular dendrimer since then[51]. Dendrimers possess several unique properties that make them a good nanoparticle platform for antimicrobial drug delivery. The highly-branch nature of dendrimers provides enormous surface area to size ratio and allows greater reactivity with microorganisms *in vivo*. In addition, both hydrophobic and hydrophilic agents can be loaded into dendrimers. Hydrophobic drugs can be loaded inside the cavity in the hydrophobic core, and hydrophilic drugs can be attached to the multivalent surfaces of dendrimers through covalent conjugation or electrostatic interaction[52, 53].

Moreover, by using antimicrobial drugs as a building block, the synthesized dendrimers themselves can become a potent antimicrobial. Dendrimer biocides are such example that contains quaternary ammonium salts as functional end groups. Quaternary ammonium compounds (QACs) are antimicrobial agents that disrupt bacterial membranes. Dendrimer biocides have displayed greater antimicrobial activity against target bacteria than small drug molecules because of a high density of active antimicrobials present on the dendrimer surfaces. The polycationic structure of dendrimer biocides facilitates the initial electrostatic adsorption to negatively charged bacteria. The absorption then increases membrane permeability and allows more dendrimers entering the bacteria, leading to leakage of potassium ions and eventually complete disintegration of the bacterial membrane[54].

The examples of these three nanoparticle platforms for antimicrobial drug delivery are summarized in Table 1.2.
<table>
<thead>
<tr>
<th>Nanoparticle Platform</th>
<th>Drug</th>
<th>Targeted Microorganism</th>
<th>Activity</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poly (D,L-lactide) (PLA) Nanospheres</td>
<td>arjunglucoside</td>
<td>Leishmania donovani</td>
<td>reduced toxicity</td>
<td>[55]</td>
</tr>
<tr>
<td>Poly lactic-co-glycolic acid (PLGA) nanoparticles</td>
<td>Phosphorothioate antisense oligonucleotide</td>
<td>HIV</td>
<td>Protection of oligonucleotides from degradation</td>
<td>[56]</td>
</tr>
<tr>
<td>Poloxamer 188 coated poly(epsilon-caprolactone) (PCL) nanosphere</td>
<td>Amphotericin B</td>
<td>Candida albicans</td>
<td>Lower in vivo toxicity due to reduced accumulation in kidney and liver</td>
<td>[57]</td>
</tr>
<tr>
<td>Polyethylene glycol (PEG)-PLA nanocapsule</td>
<td>halofantrine</td>
<td>Plasmodium berghei</td>
<td>Prolonged circulation half-life</td>
<td>[58]</td>
</tr>
<tr>
<td>stearic acid SLN</td>
<td>rifampicin, isoniazid, pyrazinamide</td>
<td>Mycobacterium tuberculosis</td>
<td>1) increased residence time 2) increased drug bioavailability 3) decreased administration frequency</td>
<td>[59]</td>
</tr>
<tr>
<td>stearic acid, soya phosphatidylcholine, and sodium taurocholate SLN</td>
<td>ciprofloxacin hydrochloride</td>
<td>gram-negative bacteria, gram-positive bacteria, and mycoplasma</td>
<td>prolonged drug release</td>
<td>[40]</td>
</tr>
<tr>
<td>stearic acid, soya phosphatidylcholine, and sodium taurocholate SLN</td>
<td>tobramycin</td>
<td>Pseudomonas aeruginosa</td>
<td>increased drug bioavailability</td>
<td>[60]</td>
</tr>
<tr>
<td>Polyamidoamine (PAMAM) dendrimers</td>
<td>Nadifloxacin and prulifloxacin</td>
<td>Various bacteria</td>
<td>Improved water solubility</td>
<td>[61]</td>
</tr>
<tr>
<td>Niclosamide</td>
<td></td>
<td>Tapeworm</td>
<td>1) Improved water solubility 2) Controllable drug release</td>
<td>[62]</td>
</tr>
<tr>
<td>Silver salts</td>
<td></td>
<td></td>
<td>1) High payload 2) Prolonged circulation half-life</td>
<td>[63]</td>
</tr>
</tbody>
</table>
1.3 Challenges in Liposomal Antimicrobial Drug Delivery

Liposomes have been recognized as one of the most widely used carriers for delivering a myriad of cosmeceuticals, pharmaceuticals, and diagnostic and imaging agents, and thus, they are selected to use for the rest of this work. Although they have many advantages toward drug delivery purpose, the applications of liposomes are usually limited by their instability. Liposomes, particularly with sub-100 nm size, are prone to fuse with one another to reduce their surface tension, leading to payload loss or undesired mixing [64-67]. Moreover, the resulting liposomes with a size much larger than 100 nm are unlikely to transport through the skin, therefore significantly diminishing their use as a dermal drug delivery vehicle [68, 69].

A few strategies have been employed to overcome this problem aiming at improving the use of liposomes as a potent delivery nanocarrier [70-73]. One extensively used approach is to coat liposome surface with a “stealth” material to enhance their in vivo stability or targeting ligands to enable preferential delivery of liposomes [74, 75]. For example, polyethylene glycol (PEG) has been frequently conjugated to liposome surface to create a stealth layer that prolongs the circulation lifetime of liposomes in the blood stream. Specifically, the PEG coating forms a hydration layer that retards the reticuloendothelial system (RES) recognitions of liposomes through sterically inhibiting hydrophobic and electrostatic interactions with plasma proteins.
However, the PEG layer not only prevents liposomes from fusing with one another but also enhances their \textit{in vivo} circulation lifetime by suppressing plasma proteins from adsorbing onto the liposome surface. The success of PEGylated liposomes has led to a group of clinically approved therapeutic products for systemic drug delivery, including Doxil, AmBisome, DepoCyt and Visudyne [76, 77]. Although the polymer-coated liposomes have shown great success for systemic drug delivery, they are less frequently used for drug delivery to treat bacterial infections. This is because the polymer coating will not only stabilize liposomes against fusion with one another but also prevent them from fusing with bacterial membranes, to which the antimicrobial payloads will be delivered. It is worth noting that bacteria usually interact with vesicular drug nanocarriers such as liposomes in a different manner from host cells or cancerous cells. The cells can internalize the entire liposomes through endocytosis while the bacteria preferentially go through membrane-membrane fusion [69, 78]. Therefore, it would be desirable to develop liposomes that are stabilized against fusion with one another before they are placed at the sites of action including the manufacturing and storage periods, while their fusion activity will be reinstalled once they are administered onto the target sites.

Here we report a novel technique to stabilize liposomes by allowing the adsorption of gold nanoparticles to the surface of liposomes and controllably release encapsulated depending on conditions that microorganism reside in. For example, it has been well documented that human skin is typically acidic (pH=3.9~6.0) [79], especially the infectious lesions on the skin [80]. The pH value is about 4.0 at the
acne lesions[81] and 4.5-6.3 at comedones [82]. Therefore acid-responsive liposomes with tunable fusion ability will be practically demanded for drug delivery to treat microorganism reside on skin or in hair follicle, such as Propionibacterium acnes (P. acnes). In this system, liposomes are stabilized with gold nanoparticles to provide stability and prevent undesirable fusion at neutral pH value during drug storage. However, when they are applied to acne lesion with pH < 5, gold nanoparticles will fall off from liposomal membrane, allowing the fusion activity of liposome and the targeted bacteria. Another strategy is to utilize virulence factors secreted from bacteria to trigger drug release at infectious sites. Alpha toxin (α-toxin) is one of virulence factors secreted from Staphylococcus aureus (S. aureus) bacteria that can invade host cells by forming pores on cell membrane, leading to uncontrolled permeation of ion and small molecules, and eventually cell lysis. Due to the tremendous availability of bacterial toxins at infection sites and their pore forming activities, these invasive molecules can be used to selectively form pores and release antimicrobials from nanoparticle-stabilized liposomes. These strategies allow liposome stabilization and smart release of drugs at the infectious sites to kill targeted bacteria.

Chapter 1 is based, in part, on the material as it appears in Current Medicinal Chemistry, 2009, Dissaya Pornpattananangkul, Che-Ming Hu, and Liangfang Zhang. The dissertation author was the primary investigator and co-author of this paper. The remainder of this dissertation will focus on stimuli-responsive liposome to address the aforementioned challenges in liposomal antimicrobial drug delivery.
Chapter 2 Stimuli-Responsive Liposomes

2.1 Acid-Responsive Liposomes

2.1.1 Introduction

Liposomes are spherical lipid vesicles with a bilayered membrane structure consisting of amphiphilic lipid molecules. They have been recognized as one of the most widely used carriers for delivering therapeutic agents. Liposomes can carry both hydrophilic and hydrophobic agents with high efficiency and protect them from undesired effects of external conditions. Their surface can be readily functionalized with specific ligands that target liposomes and their payloads to the sites of action. In addition, the composition, size, surface charge and other formulation properties of
liposomes can be well controlled to meet the needs of specific circumstances [77, 83-85]. However, their main limitation during manufacturing and storage is their instability. They fuse with one another to reduce their surface tension, resulting in payload loss, undesired mixing, and larger size. As a result, their advantages as a nano-scale drug carrier and fusion activity to interact with targeted bacteria significantly decrease [64-67].

One extensively used strategies to overcome this problem is to coat liposome surface with polyethylene glycol (PEG) [74, 75]. However, PEG will not only prevents liposomes from fusing with one another, but also prevent them from fusing with bacterial membranes. Thus, this strategy is less likely to be useful for antimicrobial drug delivery. Therefore, our goal in this study is to develop liposomes that are stabilized against fusion with one another during storage period, while their fusion activity will be resumed at target sites.

Here we report a stimuli-responsive novel gold nanoparticles-stabilized liposome system in which small gold nanoparticles (diameter: ~4 nm) bind to the surface of liposomes (diameter: sub-100 nm) and thus stabilize the liposomes at neutral pH. The bound gold particle stabilizers detach from the liposomes when the environment acidity increases to pH<5, resulting in the formation of bare liposomes that can actively fuse with various biological membranes. It has been well documented that human skin is acidic (pH=3.9~6.0) [79], especially the infectious lesions on the skin [80]. Recently, Granick et al. have reported that binding small polystyrene particles (diameter: ~20 nm) to the surface of zwitterionic liposomes
(diameter: ~200 nm) can stabilize liposomes against fusion.[67, 86, 87] However, no study to the best of our knowledge has been reported to develop stimuli-responsive nanoparticle-stabilized liposomes for possible drug delivery applications.

The principle of this study, applying carboxyl-modified gold nanoparticles to mediate the fusion activity of phospholipid liposomes, is illustrated in Figure 2.1.1. With a pKa≈5,[88] the carboxylic group is deprotonated at pH=7 resulting in negatively charged Au-COO\(^-\) nanoparticles, which can bind to cationic liposomes through electrostatic attraction and thus stabilize the liposomes. When the environment pH drops to below 5, the carboxylic group will be protonated. The resulting neutral Au-COOH nanoparticles will detach from the liposome surface due to the lack of binding forces, thereby freeing the liposomes. Gold nanoparticles are selected for this study because of their fluorescence quenching properties that can be employed to indicate their binding and detaching process and extent when a small fraction of fluorescent dyes is doped into the liposome membranes. Moreover, gold is a biocompatible noble metal[89] with antimicrobial activity against a wide type of bacteria.[90]
Figure 2.1.1. Schematic illustrations of carboxyl modified gold nanoparticles (AuC)-stabilized liposome and its destabilization at acidic pH. The liposome is stabilized by deprotonated AuC (Au-COO\(^-\)) at neutral pH. When pH drops below the pKa value of carboxylic group (pKa ~ 5), Au-COO\(^-\) are protonated to form Au-COOH, which subsequently detach from the liposome, resulting in the formation of bare liposome with fusion activity resuming.

2.1.2 Experimental Methods

2.1.2.1 Materials

Hydrogenated L-a-Phosphatidylcholine (Egg PC), 1,2-di-(9Z-octadecenoyl)-3-trimethylammonium-propane (DOTAP), Phytosphing and 1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine-N-lissamine rhodamine B sulfonyl (DMPE-RhB), and C\(_6\)-NBD were purchased from Avanti Polar Lipids, Inc. Lauric acic (LA) was obtained from Sigma Aldrich (St Louis, MO). In order to prepare carboxyl functionalized gold nanoparticles (AuC), the following chemicals were purchased: hydrogen tetrachloroaurate (HAuCl\(_4\)) (ACROS Organics), Sodium borohydride (NaBH\(_4\)) (ACROS Organics), and 3-Mercaptopropionic acid (MPA) (Sigma Aldrich).
Potassium hydrogen phthalate and potassium phosphate monobasic were purchased from EMD and Sigma Aldrich, respectively, in order to prepare buffer solutions.

2.1.2.2 Preparation of carboxyl-modified gold nanoparticles (AuC)

AuC were prepared by sodium borohydride reduction method described in full details elsewhere.[91, 92] Briefly, aqueous solution of HAuCl₄ (10⁻⁴M, 50 mL) was reduced by 0.005 g of NaBH₄ at ice cold temperature, resulting in the formation of bare gold nanoparticles (AuB). AuB were functionalized with carboxyl groups by overnight incubation with MPA (4x10⁻⁴M). The resulting AuC were washed 3 times by an Amicon Ultra-4 centrifugal filter with a molecular weight cut-off of 10 kDa (Millipore, Billerica, MA) and suspended in aqueous solution at pH=6.8.

2.1.2.3 Preparation and characterization of liposomes and AuC-liposomes

Cationic liposomes consisting of Egg PC (zwitterionic phospholipid) and DOTAP (cationic phospholipid) were prepared through the well-known extrusion method.[93] Briefly, 1.5 mg of Egg PC and DOTAP mixture (weight ratio=9:1) were dissolved in 1 mL of chloroform. The solvent was evaporated by blowing argon gas over it for 15 min. Then the dried lipid films were hydrated with 3 mL of deionized water, followed by vortexing for 1 min and sonicating for 3 min in a bath sonicator (Fisher Scientific FS30D) to produce multilamellar vesicles (MLVs). A Ti-probe (Branson 450 sonifier) was used to sonicate the MLVs for 1-2 minutes at 20 W to
produce unilamellar vesicles. To form narrowly distributed small unilamellar vesicles (SUVs), the solution was extruded through a 100 nm pore-sized polycarbonate membrane for 11 times. AuC-stabilized liposomes (AuC-liposomes) were prepared by mixing liposomes and AuC nanoparticles at desired molar ratios under gentle bath sonication for 10 min.

The hydrodynamic size and surface zeta potential of the prepared liposomes and AuC-liposomes were assessed by using the Malvern Zetasizer ZS (Malvern Instruments, UK). The mean diameter and zeta potential were determined through dynamic light scattering (DLS) and electrophoretic mobility measurements, respectively. All characterization measurements were repeated three times at 25°C. The morphology and structure of the AuC-liposome were characterized by a Hitachi HD2000 scanning transmission electron microscope (STEM) equipped with a cold cathode field emission electron source and a turbo-pumped main chamber. Samples for STEM characterization were prepared by dispersing a solution containing the AuC-liposome onto the surface of a carbon film coated Cu grid. The samples were air-dried, and then coated with a thin amorphous carbon film by evaporation. All images were recorded in the STEM as scanned beam images, using the secondary electron signal, which provides surface topology detail, the direct transmitted electron beam (unscattered electrons) or the diffracted transmission electrons collected on an annular dark field detector.
2.1.2.4 Fluorescence quenching and recovery studies

DMPE-RhB labeled liposomes were prepared by mixing 0.5 mol% DMPE-RhB with Egg PC and DOTAP prior to liposome preparation. To monitor the quenching effect of AuC on the fluorescently labeled liposomes, AuC were mixed with the liposomes at desired molar ratios (M_{AuC}/M_L) ranging from 0 to 280, followed by 10 min sonication. The fluorescence emission spectra of DMPE-RhB in the range of 500-650 nm were measured by using a fluorescent spectrophotometer (Infinite M200, TECAN, Switzerland) at an excitation wavelength of 470 nm. The emission peak at 590 nm was selected to quantify the fluorescence quenching yield.

To study fluorescence recovery yield of DMPE-RhB labeled AuC-liposome at different pH values, the AuC-liposome solution with a M_{AuC}/M_L=200 was selected. The DMPE-RhB labeled AuC-liposome were adjusted to desired pH values using proper buffer solutions with target pH values (potassium hydrogen phthalate buffer for pH=3-5, and potassium phosphate monobasic buffer for pH=5.5-7). The actual pH value of each AuC-liposome solution was measured by an Orion 3-star plus portable pH meter. The salt concentration of each AuC-liposome solution after pH adjustment was 5 mM. The fluorescence emission spectra of DMPE-RhB were measured as previously described. The mixtures of fluorescently labeled liposome and bare gold nanoparticles (AuB, no carboxyl modification) at the same molar ratios were used as positive controls.
2.1.2.5 The UV-vis absorption spectra of Auc-liposomes at pH=7 and 4

AuC-liposome were prepared following the protocol described above. To adjust the pH value of the AuC-liposome solution to pH=4, 0.1 M HCl was used because it did not induce any undesirable UV absorption background. Unbound AuC were removed from the solution by centrifugation at $1.3 \times 10^4$ rpm for 10 min. Absorption spectra in the range of 300 nm to 700 nm were recorded by a spectrophotometer. To exclude possible UV absorption from the cationic liposomes and background, free liposomes (without AuC addition) at the same concentration and pH value as the AuC-liposome were measured, whose signal was subtracted from the measured AuC-liposome UV absorption spectra. All measurements were repeated three times.

2.1.2.6 AuC-liposome fusion studies

To investigate the fusion activity of AuC-liposome against other liposomes or target cells at different pH values, negatively charged liposomes consisting of Egg PC and lauric acid (weight ratio=9:1) were synthesized by extrusion method as described above to mimic negatively charged cells. These anionic liposomes were labeled with a fluorescence resonance energy transfer (FRET) pair of chromophores, a fluorescent donor (C$_6$NBD, 0.1 mol%) and a fluorescent quencher (DMPE-RhB, 0.5 mol%). AuC-cationic liposomes ($M_{AuC}/M_L=200$) solutions were prepared and adjusted to pH=7 and pH=4, respectively. Unbound AuC nanoparticles were removed by centrifugation at $1.3 \times 10^4$ rpm for 10 min. The supernatants of the AuC-cationic
liposomes were mixed with FRET-labeled anionic liposomes with a molar ratio of 7:1. Consequently, fluorescence emission spectra at the range of 500-650 nm were obtained by exciting the samples at 470 nm using a fluorescent spectrophotometer. AuB-cationic liposome mixtures at the corresponding molar ratios and pH values were used as positive controls. The FRET-labeled anionic liposomes alone (without the addition of cationic liposomes) at the corresponding concentrations and pH values were used as negative controls. All measurements were carried out at 25°C and repeated three times.

2.1.3 Results and Discussion

We first prepared carboxyl-modified gold nanoparticles (AuC) stabilized liposomes (AuC-liposome). In the study, cationic phospholipid liposomes consisting of 90 wt% hydrogenated L-a-Phosphatidylcholine (Egg PC) and 10 wt% 1,2-di-(9Z-octadecenoyl)-3-trimethylammonium-propane (DOTAP) were prepared through the well-known extrusion method [93]. Dynamic light scattering (DLS) measurements showed the size and surface zeta potential of the formed liposomes were 88.0±1.0 nm and 24.9±2.3 mV, respectively (Figure 2.1.2).
Figure 2.1.2. Characterization of AuC-liposome by dynamical light scattering. (A) The size (diameter, nm) and (B) surface zeta potential (mV) of bare liposomes and AuC-liposome with an AuC/liposome molar ratio of 200/1.

The positive zeta potential value indicates the incorporation of DOTAP to the liposome membrane. In a separate reaction, AuC nanoparticles were synthesized following a previously published protocol [91, 92], resulting in AuC with a nearly uniform size of ~4 nm measured by scanning transmission electron microscope (STEM) (Figure 2.1.3) and a negative surface zeta potential of -25.6±4.2 mV determined by DLS. The synthesized cationic liposomes and AuC nanoparticles were then mixed with a molar ratio of 1:200 under gentle bath sonication for 10 min to form AuC-liposome. The excess AuC in the solution were removed by 10 min centrifugation at 1.3 x 10^4 rpm to ensure the subsequent particle size and surface zeta potential measurements were solely from the AuC-liposome but not from unbound
AuC particles. DLS data showed that the size of the AuC-liposome was 92.9±1.3 nm and the surface zeta potential was -25.3±0.7 mV (Figure 2.1.2).

Figure 2.1.3. Representative scanning transmission electron microscope (STEM) images showing the structure of AuC-liposome. (A) Secondary electron image shows that AuC nanoparticles adsorb on liposome surface. (B) Transmitted electron image of region shown in (A) further confirms the binding of AuC nanoparticles on liposome. (C) Dark field transmission image of AuC nanoparticles. (D) Transmission image of AuC nanoparticles.
The measured AuC-liposome size was slightly larger than that of bare liposomes because of the adsorption of 4 nm AuC nanoparticles, while the change of zeta potential from 24.9 mV to -25.3 explicitly suggests the binding of negatively charged AuC to the positively charged liposomes. The morphology and structure of the AuC-liposome were further imaged by STEM. As shown in Figure 2.1.3 AB, individual AuC particles were visible on the surface of liposomes after they were deposited on a TEM grid. Using the energy dispersive x-ray (EDX) spectrometer on the STEM, we were able to identify elementally that certain regions in Figure 2.1.3 AB contained Au and other regions contained only elements found in the liposome such as carbon and phosphorus. The size of dehydrated liposomes was larger than the size of hydrated liposomes measured by DLS due to the collapse of liposomes from a 3 dimensional sphere to a 2 dimensional thin layer.

To further confirm the binding of AuC nanoparticles to the liposome surface, a fraction of fluorescently labeled lipid, 1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine-N-lissamine rhodamine B sulfonyl (DMPE-RhB, Excitation/Emission=550 nm/590 nm), was doped into the liposome membranes. It was expected that AuC binding would quench the fluorescence dye underneath or nearby the AuC particles because of a fluorescence resonance energy transfer (FRET) mechanism. AuC nanoparticles were mixed with fluorescently labeled liposomes with a molar ratio \( \frac{M_{AuC}}{M_L} \) ranging from 0 to 280. Fluorescence emission intensity at 590 nm was recorded and quenching yield was calculated as following: quenching yield \( (\%) = (1 - \frac{I_{AuC-L}}{I_L}) \times 100 \), in which \( I_{AuC-L} \) and \( I_L \) represent the fluorescence
intensity of RhB-labeled liposomes in the presence and absence of AuC nanoparticles, respectively. As shown in Figure 2.1.4A, when $M_{\text{AuC}}/M_L$ molar ratio increased, the quenching yield raised and reached 100% at $M_{\text{AuC}}/M_L=280$. Since the diameters of liposomes and AuC nanoparticles were about 88 nm and 4 nm, respectively, the surface coverage of AuC on liposome surface was about 14% at the $M_{\text{AuC}}/M_L$ ratio of 280:1 if assuming all AuC attached to liposome surface. According to the FRET mechanism, the adsorbed AuC particles can effectively quench DMPE-RhB probes not only underneath the AuC but also within 2~5 nm region surrounding the AuC particles. This will result in a near 100% theoretical quenching yield, which is consistent with what has been observed in Figure 2.1.4A. Although more AuC particles might be able to adsorb onto the ~86% unoccupied liposome surface, further studies demonstrated that the quenching yield remained as a plateau of 100% when more AuC were added into the solution above the fully quenching point of $M_{\text{AuC}}/M_L=280$. Figure 2.1.4A inset showed the representative fluorescence emission spectra of the AuC-liposome in the range of 500–650 nm at different $M_{\text{AuC}}/M_L$ ratios with an excitation wavelength of 470 nm. We found that this excitation wavelength can effectively excite DMPE-RhB probe doped in liposome membranes while minimally interfering with the fluorescence emission spectra.

Next we investigated the acid-responsive detachment of AuC from the liposomes. Hypothetically, when the environment pH value is reduced below the pKa value of carboxylic acid, the negatively charged Au-COO$^-$ will be protonated to form neutral Au-COOH, which may detach from the cationic liposomes due to the
elimination of electrostatic attraction. Subsequently, the detaching of AuC will induce a fluorescence recovery of the DMPE-RhB probes doped in the liposomes. To test this hypothesis, AuC-liposome solution with a $M_{\text{AuC}}/M_L$ ratio of 200 was used to study relative fluorescence recovery yield of DMPE-RhB at various pH values. The pH of the AuC-liposome solution was adjusted to desired values ranging from pH=7 to pH=3 using buffer solutions consisting of potassium hydrogen phthalate or potassium phosphate monobasic with a final salt concentration of 5 mM. Fluorescence emission intensity at 590 nm of the AuC-liposome solutions at various pH values was recorded. Considering the detached AuC nanoparticles suspending in the fluorescently labeled liposome solutions may quench the DMPE-RhB dyes as well through random collision, we used relative recovery yield to describe the fluorescence recovery upon pH change. The fluorescence intensity of AuC-liposome at each pH point was normalized with that of liposomes mixing with the same amount of bare gold nanoparticles (AuB), which are neutral particles without carboxyl modification and characteristic of Au-COOH. The relative recovery yield was defined as following: Relative recovery yield (%)=$I_{\text{AuC-L}}/I_{\text{AuB-L}} \times 100$, in which $I_{\text{AuC-L}}$ and $I_{\text{AuB-L}}$ represent fluorescent intensity of AuC stabilized liposomes and mixture of liposomes and AuB at the same concentration as AuC-liposomes at various pH values. As shown in Figure 2.1.4B, the relative recovery yield of DMPE-RhB labeled AuC-liposome slightly decreased from 23% to 18% when the pH value decreased from 7 to 5.5. Then it dramatically increased from 18% to about 55% when the pH value further decreased from 5.5 to 3. The slight decrease of the relative recovery
yield from pH=7 to 5.5 indicates that more AuC particles adsorb onto the liposomes or stronger binding between AuC and liposomes occurs at pH=5.5 than at pH=7. This might be because cationic lipid DOTAP becomes more positively charged at lower pH resulting in stronger charge-charge attraction between AuC and the liposomes. While when the pH value was less than 5.5 within the range of 5.5~3, the protonation effect of AuC was more dominant than any other effects, which significantly weakened the electrostatic attraction. Therefore, AuC detached from the liposome surface leading to high fluorescence recovery. Figure 2.1.4B inset showed the representative fluorescence emission spectra of the AuC-liposome in the range of 500–650 nm at different pH values ranging from 7 to 3 with an excitation wavelength of 470 nm. These fluorescence recovery results are consistent with the surface zeta potential measurements of the AuC-liposome at different pH values. We found that the surface zeta potential of the AuC-liposome increased from -25.3±0.7 mV at pH=7 to +30.1±2.1 mV at pH=4, indicating the detachment of the AuC from the liposome surface at acidic pH. The surface zeta potential of the AuC-liposome at pH=4 is slightly higher than bare liposomes at pH=7, 24.9±2.3 mV (Figure 2.1.2B), which may be because the cationic lipid DOTAP is more positively charged at acidic pH.
Figure 2.1.4. Fluorescence quenching and recovery yields of AuC-liposome at different AuC/liposome molar ratios ($M_{\text{AuC}}/M_L$) and different pH values. (A) AuC nanoparticles at different $M_{\text{AuC}}/M_L$ molar ratio are allowed to adsorb to fluorescently labeled liposomes. Percentages of fluorescence quenching yield are plotted against $M_{\text{AuC}}/M_L$ ratio. Inset: fluorescence emission spectra of AuC-liposome at different $M_{\text{AuC}}/M_L$ ratio (from top to the bottom: 0, 22, 44, 66, 88, 110, 132, 154, 176, 200, 220, 240, 260, and 280). (B) Relative fluorescence recovery yield of AuC-liposome ($M_{\text{AuC}}/M_L = 200$) at different pH values. Inset: fluorescence emission spectra of AuC-liposome at a series of pH values (from top to the bottom: 3, 3.5, 4, 4.5, 5, 7, 6.5, 6, and 5.5).

The binding of AuC to liposome surface at neutral pH and detaching at acidic pH were further examined by measuring UV-vis absorption of AuC-liposome at pH=7 and pH=4, respectively, after the removal of unbound AuC via proper centrifugation. Here HCl was used to adjust the pH of the AuC-liposome solutions instead of using buffer solutions because some UV absorption of the buffer was detected. After incubating the AuC-stabilized cationic liposomes (not fluorescently
labeled) with HCl for 10 min at pH=7 and pH=4, respectively, the AuC-liposome solutions were centrifuged to precipitate unbound AuC nanoparticles. The UV-vis absorption spectra of the resulted supernatants were then recorded in the range of 300 nm to 700 nm as shown in Figure 2.1.5. At pH=7, UV absorption spectrum of AuC was clearly detected but not at pH=4. The observed UV absorption spectra were consistent with the color difference of the supernatant as shown in Figure 2.1.5 inset. At pH=7, a small amount of particle precipitates was observed while the color of the supernatant remained as red, characteristic of gold nanoparticles. In contrast at pH=4, a large amount of particle precipitates appeared and the color of the supernatant became clear. This clear supernatant was then subjected to measuring the size and surface zeta potential using DLS with results similar as bare liposomes. These data suggest that when the pH value (e.g. pH=7) was higher than the pKa (~5) of carboxylic acid, AuC were in deprotonated form (Au-COO⁻) and thus strongly bound to cationic liposomes. So they could not be separated from liposomes by centrifugal force. However, when the pH value (e.g. pH=4) was less than the pKa value, AuC were protonated to Au-COOH form which no longer adsorbed on the liposomes. The unbound Au-COOH particles were readily separated from the solution by centrifugation.
Figure 2.1.5. UV-vis absorption spectra of AuC-liposome at pH=7 (red solid line) and pH=4 (black dash line), respectively, after removal of unbound AuC through centrifugation. At pH=7, clear UV absorption spectrum of AuC was detected, indicating the strong binding of deprotonated AuC on liposome surface. At pH=4, negligible UV absorption of AuC was detected, indicating the detaching of protonated AuC from the liposome surface. Inset: AuC-liposome solutions after centrifugation to remove free AuC. Red color indicates the presence of AuC in the solution at pH=7.

After having demonstrated the binding and detaching of AuC nanoparticles from cationic liposomes upon environment acidity changes, we finally examined the controllable fusion activity of the liposomes mediated by the AuC nanoparticles. To this end, we prepared anionic liposomes consisting of Egg PC and lauric acid (LA), which were mixed with AuC-stabilized cationic liposomes at different pH values. It was expected that bare cationic liposomes would bind to and fuse with anionic liposomes intimately after the AuC were protonated and detached from the cationic liposomes. To monitor the fusion process and the fusion extent, the anionic liposomes
were pre-labeled with a FRET pair of chromophores, and the change in FRET signal was measured upon mixing the FRET-labeled anionic liposomes with AuC-stabilized cationic liposomes at pH=7 and pH=4, respectively. FRET is a widely used technique that precisely measures the distance of two subjects at the molecular level based on an energy transfer mechanism of two chromophores [94]. When the two chromophores are in close proximity (<10 nm), excited donor can transfer energy to the acceptor through a nonradiative long-range dipole-dipole coupling mechanism. Here we incorporated a fluorescence donor (C<sub>6</sub>NBD: excitation/emission=470 nm/520 nm) and a fluorescence acceptor (DMPE-RhB: excitation/emission=550 nm/590 nm) into the lipid membranes of anionic liposomes. By controlling the molar ratio between the donor and the acceptor, we prepared the fluorescent anionic liposomes in which the fluorescence emission from the donor was completely quenched by the acceptor [95]. We hypothesized that if the anionic liposomes fuse with the cationic liposomes, the spread of the donor and acceptor chromophores within the cationic liposomes will alleviate or eliminate the FRET efficiency, resulting in fluorescence recovery of the donor.

For this fusion study, AuC-stabilized cationic liposomes (M<sub>AuC</sub>/M<sub>L</sub>=200) were first adjusted to pH=7 and pH=4, respectively, using buffer solutions. The resulting unbound AuC nanoparticles were removed from the solutions via 10 min centrifugation at 1.3x10<sup>4</sup> rpm in order to eliminate fluorescence quenching effect of free AuC in the solutions through random collision. Subsequently, the cationic liposomes were mixed with the FRET-labeled anionic liposomes at a molar ratio of
7:1. The mixtures were then excited at the wavelength of 470 nm and fluorescence emission spectra in the range of 500-650 were recorded as shown in Figure 2.1.4A. Since the fluorescent receptor DMPE-RhB was also excited at the 470 nm resulting in a dominant emission peak at 590 nm, we zoomed in to the 500-540 nm emission window which was predominantly from the C₆NBD (Figure 2.1.6B). We found that significant fluorescence recovery of C₆NBD occurred at pH=4 as compared to at pH=7. The most plausible explanation is that at pH=7 Au-COO⁻ nanoparticles strongly bind to the cationic liposomes and prevent them from fusion with anionic liposomes. However, at pH=4 the protonated Au-COOH nanoparticles detach from the cationic liposomes, resulting in bare cationic liposomes that effectively fuse with the anionic liposomes. To rule out the possibility that pH adjustment will affect the FRET efficiency within the anionic liposomes, FRET-labeled anionic liposomes adjusted to the corresponding pH values and concentrations without mixing with cationic liposomes were applied as negative controls. When the control samples were excited at 470 nm, no considerable fluorescence emission difference at 530 nm was detected at pH=7 and pH=4. Additionally, AuB nanoparticles (neutral and no carboxyl modification) were used as positive controls. Strong fluorescence emission of C₆NBD at 530 nm appeared at both pH=7 and pH=4, indicating that AuB do not bind tightly to the cationic liposomes to prevent them from fusion with the anionic liposomes at both neutral and acidic pH values. Figure 2.1.6C highlighted the relative fusion efficiency of AuC-cationic liposomes with anionic liposomes over AuB-cationic liposomes with anionic liposomes, taking anionic liposomes alone at the
corresponding pH values and concentrations as background. The relative fusion ability at different pH values were calculated as following: Relative fusion (%)=\( \frac{I_{530,\text{AuC}} - I_{530,\text{H2O}}}{I_{530,\text{AuB}} - I_{530,\text{H2O}}} \times 100 \), in which \( I_{530,\text{AuC}} \) represents fluorescence emission intensity at 530 nm of the AuC-cationic liposomes mixing with the anionic liposomes; \( I_{530,\text{AuB}} \) represents fluorescence emission intensity at 530 nm of the AuB-cationic liposomes mixing with the anionic liposomes; \( I_{530,\text{H2O}} \) represents fluorescence emission intensity at 530 nm of the anionic liposomes alone. As shown in Figure 2.1.6C, the relative fusion yield of AuC-cationic liposomes was 24.4±1.6 at pH=7 and 81.1±1.2 at pH=4, indicating the feasibility of using AuC to mediate the fusion activity of liposomes.
Figure 2.1.6. FRET measurement of AuC-mediated liposome fusion at pH=7 and pH=4, respectively. A fluorescent donor (C₆NBD) and a fluorescent quencher (DMPE-RhB) were simultaneously incorporated into the anionic liposomes with a proper molar ratio that the quencher effectively quenched the fluorescence emission from the donor. The FRET-labeled anionic liposomes were then mixed with AuC-cationic liposomes. (A) Fluorescence emission spectra of C₆NBD and DMPE-RhB with an excitation wavelength of 470 nm. Red lines represent AuC-cationic liposomes mixing with the anionic liposomes at pH=7 (solid line) and pH=4 (dashed line), respectively. Black lines represent AuB-cationic liposomes mixing with the anionic liposomes at pH=7 (solid line) and pH=4 (dashed line), respectively. Grey lines represent an aqueous solution of the anionic liposomes alone without any gold nanoparticles or cationic liposomes at pH=7 (solid line) and pH=4 (dashed line), respectively. (B) A zoom in of fluorescence emission spectra of C₆NBD (donor) at different conditions from panel (A). (C) Relative fusion activity of AuC-cationic liposomes with anionic liposomes in contrast to AuB-cationic liposomes at pH=7 and pH=4, respectively.
2.1.4 Conclusions

In conclusion, phospholipid liposomes with acid-responsive stability and fusion activity were formed by attaching carboxyl-modified gold nanoparticles to the outer surface of cationic liposomes. At neutral pH, the negatively charged gold nanoparticles bound to the surface of cationic liposomes (diameter: ~ 90 nm; surface zeta potential: ~ +25 mV), resulting in a slight size increase and a dramatic surface charge change to ~ -25 mV. The adsorbed gold nanoparticles effectively quenched the fluorescent dyes doped in the liposome membranes with a quenching yield up to 100%. In contrast at acidic pH values (e.g. pH<5), the gold nanoparticles detached from the liposome membranes at an extent depending on the environment acidity, resulting in fluorescence recovery of the dyes. The binding and detaching of gold nanoparticles from the liposomes were further confirmed by UV-vis absorbance measurements. It was also demonstrated that the adsorption of gold nanoparticles can freeze the liposomes from fusing against one another, while the fusion activity of liposomes resume at acidic environments due to the detaching of gold particle stabilizers. We speculate that similar strategy can be generalized to anionic liposomes using amine-modified gold nanoparticles, which will be stable at neutral condition but destabilized in basic environments in which the amine will be deprotonated. Since the stability issues of liposomes have imposed negative impacts on their medical and biological applications as a drug delivery vehicle or functional nanocontainer, this work may provide a new paradigm of using liposomes as an environment-responsive nanocarrier with controllable stability and fusion activity.
2.2 Virulence Factor-Responsive Liposomes

2.2.1 Introduction

Using nanoparticles to differentially deliver therapeutic agents to the sites of action (also called targeted drug delivery) represents a central goal, a key challenge as well, of nanomedicine research [96-98]. A common approach to reach this goal is to functionalize the surface of the nanoparticles with targeting ligands that specifically bind to the receptors over-expressed by the target cells [76, 99]. Various molecules have been demonstrated to bind to target cells including antibody, antibody fragments, aptamers, peptides, small molecules and so on [100, 101]. While great progress has been made to use ligands for active cellular targeting, none of the products have ever been approved and only three targeted nanoparticle systems are now in phase I/II clinical trials [76, 102]. This is mainly due to the complexity and off-target effect of these ligand modified nanoparticles. Herein we report an entirely new concept of targeted drug delivery to treat bacterial infections. Instead of using targeting ligands to actively target the drug carriers to the bacteria of interest, we take advantage of the bacterial virulence factors such as toxins secreted by the target bacteria and use them to trigger the release of therapeutic payloads and thus kill the bacteria. In this approach, prior to seeing the target bacteria, drugs are protected inside the nanocarriers and will not be released, thereby eliminating all adverse side effects due to premature drug leakage or non-specific drug release. As a proof-of-concept, here we demonstrate that bacterial toxins can be utilized to trigger antibiotic release from gold nanoparticle-stabilized phospholipid liposomes and the released
antibiotics can subsequently inhibit the growth of *Staphylococcus aureus* (*S. aureus*) bacteria that secrete the toxins.

This study intends to integrate the therapeutic needs to treat bacterial infections with the well-studied pore-forming activities of toxins secreted by bacteria and the recent advancements in liposome chemistry. There are a variety of molecules that possess pore-forming activity, including bacterial toxins, animal toxins, immune proteins, and synthetic compounds such as Triton X-100.[103-106] Alpha hemolysin, also named α-toxin, is one of the common toxins secreted by *Staphylococcus aureus* (*S. aureus*) bacteria as a water-soluble protein monomer with a molecular weight of 34 kDa.[107] This protein can spontaneously incorporate into lipid membranes and self oligomerize to form a heptameric structure with a central pore. The pore size is about 2 nm that allows small molecules up to 3 KDa to passively diffuse through the membranes.[108, 109] In nature, *S. aureus* bacteria secrete α-toxin that can bind to the outer membranes of susceptible cells. Upon binding, rapid pore forming facilitates uncontrolled permeation of water, ions, and small molecules, rapid discharge of vital molecules such as ATP, dissipation of the membrane potential and ionic gradients, and irreversible osmotic swelling leading to the cell lysis.[107] Considering the tremendous availability of bacterial toxins at bacterial infection sites and their pore forming activities, we hypothesize that these invasive molecules can be utilized to selectively release antimicrobials from liposomes that are stabilized by small gold nanoparticles to avoid undesirable membrane-membrane fusion and drug leakage.
This strategy allows smart release of drugs at the infectious sites to kill toxin-secreting bacteria while not producing any toxicity effects on healthy tissues.

Liposomes are spherical lipid vesicles with a bilayer membrane structure consisting of amphiphilic lipid molecules and have been studied extensively as antimicrobial drug delivery vehicles for decades due to their unique features, including highly biocompatible lipid materials, ability to deliver hydrophilic and lipophilic drugs, lipid bilayer structure that can fuse with bacterial membranes, and easy surface modification [78, 110, 111]. There are a few liposome formulations that have been approved by the Food and Drug Administration (FDA) for therapeutic purposes. For example, AmBisome (NeXstar Pharmaceuticals, San Dimas, USA) is an FDA approved liposomal formulation of amphotericin B, which has been widely used in the clinic to treat Candida spp, Aspergillus spp, Fusarium spp, and other fungi infections in neutropenic, visceral leishmaniasis, and methylmalonic acidaemia patients.[112, 113] Despite these advantageous features of liposomes as a delivery vehicle, the applications of liposomes are usually limited by their instability due to uncontrollable fusion among liposomes, leading to short shelf-life, undesirable payload loss, and unexpected mixing.[64-66] An extensively used approach to stabilize liposomes is to coat their surface with a “stealth” material such as polyethylene glycol (PEG).[74, 75] PEGylated liposomes can not only prevent liposomes from fusing with one another but also enhance their in vivo circulation lifetime by suppressing plasma proteins from adsorbing onto the liposome surface. Therefore, they have been widely used for systemic drug delivery.[77] However,
PEGylated liposomes are rarely used for topical drug delivery, especially to treat bacterial infections. This is mainly because the polymer coatings will not only stabilize liposomes against fusion but also prevent them from fusing with bacterial membranes or prevent pore forming proteins such as toxins from accessing to the liposomes to release drug payloads. Therefore it would be desirable to develop liposomes that are stabilized against fusion with synthetic or biological membranes, but they are accessible to pore forming proteins for controlled drug release once they are applied onto the target skin sites.

Herein, we synthesize a novel liposome formulation stabilized by chitosan-modified gold nanoparticles (AuChi) to differentially release a model antibiotic, vancomycin, to inhibit the growth of *S. aureus* bacteria for topical treatment of skin bacterial infections. Figure 2.2.1 illustrates the working principle of toxin-triggered antibiotic release from gold nanoparticle-stabilized liposomes for the treatment of the bacteria that secrete the toxins. The cationic AuChi bind to the negatively charged liposome surfaces through electrostatic attraction and thus stabilize liposomes against fusion with one another and avoid undesirable antibiotic leakage. When the stabilized liposomes are in the vicinity of *S. aureus* bacteria, the bacterium-secreted toxins will insert into liposome membrane and create pores, through which the encapsulated antibiotic will be released. The released vancomycin, as staying in close to the bacteria, will then exert its antimicrobial activity rapidly and locally. In the study, we test the pore forming activity and payload release kinetics of the AuChi-stabilized liposomes (AuChi-Liposome) in the presence of α-toxin and *S. aureus* bacteria.
respectively. We also demonstrate that the released antibiotics from the liposomes in the presence of *S. aureus* are sufficient to inhibit the growth of the bacteria.

**Figure 2.2.1.** Schematic principle of bacterial toxin-triggered antibiotic release from gold nanoparticle-stabilized liposomes to treat toxin-secreting bacteria. Vancomycin-loaded liposomes are protected by absorbing chitosan-coated gold nanoparticles (AuChi) onto their surface to prevent them from fusing with one another or with bacterial membranes. Once the AuChi-stabilized liposomes (AuChi-Liposome) encounter bacterial toxins, the toxins will form pores in the liposome membranes and thus release the encapsulated antibiotics, which subsequently kill or inhibit the growth of the bacteria that secrete the toxins.
2.2.2 Experimental Methods

2.2.2.1 Materials

Hydrogenated L-α-phosphatidylcholine (Egg PC) and cholesterol were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). Sephadex G-75 was purchased from Fisher Scientific (Pittsburgh, PA). 8-amino-1,3,6-trisulfonic acid disodium salt (ANTS) and p-xylene-bis-pyridinium bromide (DPX) were obtained from Invitrogen (Carlsbad, CA). Poly(ethylene glycol) methyl (Mₙ=2000 Da) and Triptic Soy Broth (TSB) were purchased from Sigma Aldrich (St Louis, MO). Hydrogen tetrachloroaurate (HAuCl₄) and sodium borohydride (NaBH₄) were from ACROS Organics (Geel, Belgium). Chitosan-50 was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

2.2.2.2 Preparation and characterization of AuChi and AuChi-Liposome

Chitosan-modified gold nanoparticles (AuChi) were prepared by a sodium borohydride reduction technique.[91, 114] Briefly, aqueous solution of HAuCl₄ (10⁻⁴ M, 50 mL) was reduced by 0.005 g of NaBH₄ at ice cold temperature to prepare bare gold nanoparticles. The acquired bare gold nanoparticles were then incubated overnight with 0.1 % w/v chitosan that was pre-dissolved in 0.1 M acetic acid. The resulting AuChi were purified 3 times by an Amicon Ultra-4 centrifugal filter with a molecular weight cut-off of 10 kDa (Millipore, Billerica, MA).
Liopsomes were prepared following a previously described extrusion method.[95] Briefly, 9 mg of lipid components were dissolved in 1 mL chloroform, and then the organic solvent was evaporated by blowing argon gas over the solution for 15 minutes to form a dried lipid film. The lipid film was rehydrated with 3 mL of deionized water with ANTS/DPX dyes or vancomycin, followed by vortexing for 1 min and sonicking for 3 min in a bath sonicator (Fisher Scientific FS30D, Pittsburgh, PA) to produce multilamellar vesicles (MLVs). Then the obtained MLVs were sonicated for 1-2 min at 20 W by a Ti-probe (Branson 450 sonifier, Danbury, CT) to produce unilamellar vesicles. The solution was extruded through a 100 nm pore-sized polycarbonate membrane for 11 times to form narrowly distributed small unilamellar vesicles (SUVs). The liposomes were purified by gel filtration with a Sephadex G-75 column equilibrated with water or isotonic PBS solution to remove unencapsulated dyes or drugs. To prepare AuChi-stabilized liposomes (AuChi-Liposome), the pH of both AuChi and liposome solutions was adjusted to 6.5 using HCl. Then the liposomes and AuChi at desired molar ratio were mixed together, followed by 10 min bath sonication, to prepare AuChi-Liposome.

UV-Vis absorbance spectrum of AuChi from 300 to 600 nm was recorded by a spectrophotometer (Infinite M200, TECAN, Mannedorf, Switzerland). The morphology of the AuChi was characterized by a scanning transmission electron microscope (STEM) equipped with a cold cathode field emission electron source and a turbo-pumped main chamber (Hitachi HD2000, Tokyo, Japan). The STEM was operated at 200 keV accelerating voltage and 20 mA current, and images were
recorded in both secondary electron mode and transmitted electron mode. Elemental analysis was performed with an EDAX energy dispersive x-ray spectrometer (EDS). Malvern Zetasizer ZS (Malvern Instruments, Worcestershire, UK) was used to measure the hydrodynamic size and surface zeta potential of the prepared AuChi, liposome, and AuChi-Liposome. The mean liposome diameter and surface zeta potential were determined by dynamic light scattering (DLS) and electrophoretic mobility measurements respectively. All characterization measurements were repeated three times at 25°C.

2.2.2.3 AuChi-Liposome stability

Liposomes, loaded with 12.5 mM of ANTS and 45 mM of DPX, were mixed with AuChi at different molar ratios (1:0, 1:150, or 1:300). The obtained AuChi-Liposome were incubated with bare liposomes, which were neither loaded with dyes nor stabilized by AuChi, at a molar ratio of 1:4 for 1 h at room temperature. The samples were then filtered through a Microcon YM-100 centrifugal filter with a molecular weight cut-off of 100 kDa (Millipore, Billerica, MA) for 20 min at 13.2 x 10³ rpm. The amount of ANTS in the filtrate was measured for its fluorescence emission intensity at 510 nm using a fluorescent spectrophotometer (Infinite M200, TECAN, Mannedorf, Switzerland) with an excitation wavelength of 360 nm.
2.2.2.4 Pore forming assay

To study the pore forming activity of α-toxin against liposomes, 12.5 mM of ANTS and 45 mM of DPX were co-encapsulated into the liposomes, at which the fluorescence of ANTS was maximally quenched by DPX. The resulting liposomes (600 µg/mL) were then incubated with α-toxin (20 µg/mL) for 1 hr at room temperature. Once the pore forms, the encapsulated dyes will leach out of the liposomes, resulting in a fluorescence recovery of ANTS. After incubation, the fluorescence emission intensity of ANTS at 510 nm was measured by using a fluorescent spectrophotometer with an excitation at 360 nm. To obtain maximal dye leakage, Triton X-100 (1% v/v) was used as a positive control to completely lyse the liposomes. ANTS/DPX loaded liposomes at the corresponding concentrations in the absence of α-toxin served as a negative control and experimental background. To determine the optimal liposome formulation, liposomes composed of Egg PC and cholesterol (0, 10 wt%, 25 wt%, 50 wt%) were prepared and loaded with ANTS/DPX dyes to test their pore forming property, respectively. The effect of PEG on liposome pore forming property was assessed by adding PEG into the liposome solutions at various PEG concentrations: 1, 25, 50, 100, or 150 mg/mL.
2.2.2.5 Toxin-triggered vancomycin release

Vancomycin (10 mg/mL) loaded liposomes were stabilized by AuChi (Vancomycin AuChi-Liposome). To measure the drug loading yield of Vancomycin-Liposome and Vancomycin AuChi-Liposome, 1 mL of the liposome solution was vacuum dried for 2 h to remove all the liquid, the pallet was then reconstituted with 500 µL water. The obtained suspension was centrifuged at 5000 rpm for 5 min and the supernatant was collected for reversed phase high performance liquid chromatography (HPLC) using Agilent 1100 series (Santa Clara, CA). Samples were injected into a Zorbax C18 column with an injection volume of 80 µL. The elution was performed with a gradient mobile phase composed of acetonitrile and water with 0.1 % (v/v) trifluoroacetic acid (TFA) (8-18% acetonitrile, 0-20 min) at a flow rate of 1 mL/min. Vancomycin was detected by a UV/Vis detector at 280 nm and the detector temperature was 20 °C. The acquired vancomycin intensity was compared with a linear standard curve of vancomycin at different concentrations to calculate the amount of vancomycin encapsulated inside the liposomal formulations.

To measure the toxin-triggered vancomycin release from the liposomes, the sample was mixed with PEG (100 mg/mL) and incubated with a methicillin-resistant Staphylococcus aureus strain, MRSA252 (1x10^8 CFU/mL), in 5 % (v/v) tryptic soy broth (TSB) at 37°C for 0.5 h and 24 h, respectively. After incubation, free vancomycin was separated by filtration through centrifugal filter unit (100 kDa MWCO) for 20 min at 13.2 x 10^3 rpm. The amount of vancomycin in filtrate was quantified by HPLC following the protocol described above.
2.2.2.6 Antimicrobial assay

Vancomycin AuChi-Liposome were mixed with PEG (100 mg/mL) and incubated with MRSA252 (1x10⁸ CFU/mL) in 5% (v/v) TSB at 37°C for 24 h. After incubation, the absorbance of the bacteria at 600 nm was measured by a spectrophotometer to determine bacterial growth. To exclude possible interference from background, the absorbance of the corresponding samples without MRSA252 was measured and subtracted from the obtained OD₆₀₀. In the study, vancomycin loaded liposome without AuChi stabilization (Vancomycin Liposome) and free vancomycin served as positive controls, while AuChi-Liposome (without vancomycin) and PBS served as negative controls. All experiments were repeated three times.

2.2.3 Results and Discussion

In order to prepare AuChi-Liposome, AuChi were first synthesized by an ex situ stabilization technique following a previously described protocol.[91, 114] Briefly, gold hydrosol was synthesized by sodium borohydride reduction method and then was stabilized by a calculated amount of chitosan in an ambient condition. The formation of AuChi was first confirmed by the ¹H-NMR spectroscopy. As shown in Figure 2.2.2A, the characteristic proton resonance of chitosan was significantly shifted towards upfield when chitosan was attached to gold nanoparticles. For example, in the spectrum of free chitosan, the protons at α-carbon (anomeric carbon, C-1) with a resonance peak at 4.8 ppm was completely masked by the broad D₂O
resonance; the protons at β-carbon (C-2 carbon) showed a resonance peak at 2.9 ppm; and all other glycosidic protons were centered at 3.3 to 3.8 ppm. In contrast, in the $^1$H-NMR spectrum of AuChi, both α and β protons were shifted from 4.8 to 4.3 ppm and 2.9 to 2.5 ppm, respectively. In addition, the broad peaks centered at 3.3 to 3.8 ppm corresponding to the glycosidic protons of chitosan were significantly shifted towards upfield and centered at 2.6 to 3.5 ppm. This significant shifting of proton towards upfield can be attributed to their close proximity to the metal center and the inhomogeneity created by metal center, which further confirms the formation of AuChi. Similar shifting of proton resonance in close proximity to the metal center has been previously observed on different amino acid capped gold nanoparticles.[91] The formation of AuChi was further confirmed by UV-Vis spectroscopy. As shown in Figure 2.2.2B, AuChi exhibit a strong absorbance at 512 nm, characteristic of the corresponding bare gold nanoparticles without chitosan coating. This indicates that the coating of chitosan did not alter the plasmon resonance of gold nanoparticles. The morphology of the AuChi particles was imaged by scanning transmission electron microscope (STEM). Secondary electron (SE) signal, which provides surface topology detail, showed ~10 nm size of AuChi with nearly uniform size distribution. The direct transmitted electron (TE) signal showed ~ 4 nm size of the inner gold core, which was consistent with the size of unmodified gold nanoparticles. Based on both the SE and TE images (Figure 2.2.2B insets), we conclude that the increase in size from 4 nm to 10 nm was solely contributed by the coating of chitosan, but not the aggregation of gold particles.
As surface properties of AuChi are crucial for their interactions with liposomes, we next characterized the surface zeta potential of AuChi by measuring their electrophoretic mobility using dynamic light scattering (DLS). The zeta potential of AuChi was 43.4 ± 1.0 mV, indicating the presence of cationic amine groups of chitosan on the particle surface. Subsequently, liposomes consisting of hydrogenated L-a-phosphatidylcholine (Egg PC) and cholesterol (50:50 weight ratio) were prepared by vesicle extrusion technique. In order to exclude the interference of ionic strength in surface zeta potential measurements, the liposomes were prepared in deionized water. The size and surface zeta potential of the formed liposomes were 110 ± 1 nm and -14.1 ± 0.4 mV, respectively (Figure 2.2.2C). Then the AuChi-Liposome were prepared by mixing the synthesized liposomes and AuChi at a molar ratio of 1:300 under gentle bath sonication for 10 min. The size and surface zeta potential of the resulting AuChi-Liposome complexes were characterized by DLS. The measured size of AuChi-Liposome was slightly larger than that of bare liposomes suggesting the adsorption of 10 nm AuChi onto the liposome surface. The surface zeta potential changed explicitly from -14.1 ± 0.4 mV to 35.6 ± 0.4 mV (Figure 2.2.2C), which confirms the binding of positively charged AuChi to the negatively charged liposomes through electrostatic attraction.
Figure 2.2.2. Synthesis and characterization of AuChi and AuChi-Liposome. (A) $^1$H-NMR spectra of chitosan and AuChi, indicating the coating of chitosan on the surface of gold nanoparticles. (B) UV-Vis absorption spectrum of AuChi. Insets: representative secondary electron image (SEI) of AuChi and transmitted electron image (TEI) of the inner gold nanoparticles of AuChi. (C) The surface zeta potential (mV) of bare liposome (without AuChi) and AuChi-Liposome with a liposome/AuChi molar ratio of 1:300.
The stability of AuChi-Liposome was evaluated by a fluorescence assay consisting of 8-aminonaphthalene-1,3,6-trisulfonic acid disodium salt (ANTS) and p-xylene-bis-pyridinium bromide (DPX). ANTS is a polyanionic fluorophore and DPX is a corresponding cationic quencher. This pair of fluorophore/quencher has been widely used to study liposomal leakage upon liposome fusion with one another or with other biological membranes and thus to evaluate the stability of liposomes.[115, 116] When these two dyes are co-encapsulated inside liposomes at a proper molar ratio, the fluorescence emission of ANTS can be maximally quenched by DPX through a collisional quenching effect. However, when the dye-loaded liposomes are not stable and fuse with other substances, the dyes will leach out of the liposomes and be diluted by the surrounding medium. The dilution will reduce the chance of collision between ANTS and DPX and then lead to fluorescence recovery of ANTS. Therefore, with an excitation at 360 nm, ANTS emission signal at 510 nm is typically used to test the stability of liposomes. For instance, Figure 2.2.3A shows the fluorescence emission signal of ANTS/DPX loaded liposomes in PBS and in the presence of 1% Triton X 100 surfactant, respectively. It was clearly seen that negligible signal from ANTS was detected when the liposomes are intact in PBS buffer, but a significant signal increase occurred in the presence of a membrane pore-forming surfactant such as Triton X-100. Herein we tested the stability of AuChi-Liposome complex at various liposome/AuChi molar ratios (e.g., 1:0, 1:150, and 1:300). The AuChi-Liposome were pre-loaded with ANTS and DPX and then each sample was incubated with bare liposomes at the molar ratio of 1:4 for 1 h. The bare
liposomes were neither stabilized with AuChi nor loaded with the dye pair. If fusion between AuChi-Liposome and bare liposomes occurs, it is expected that some of the dyes will transfer from AuChi-Liposome to bare liposomes. To amplify the signal of the transferred dyes, the sample was centrifuged through a filter membrane at 13.2 x 10^3 rpm for 20 min, at which condition both bare liposomes and unstable AuChi-Liposome were ruptured and completely released the dyes, while stable AuChi-Liposome remain intact. Therefore, the fluorescence intensity of ANTS detected in the filtrate was the accumulative signal from unstable AuChi-Liposome that have fused with either bare liposomes or filter membrane. As shown in Figure 2.2.3B, high level of ANTS signal was detected when the liposomes were not protected by any AuChi. In contrast, when the liposome/AuChi molar ratio was 1:150 and 1:300, the detected ANTS signal was only 30% and 20%, respectively, of the bare liposomes. The obtained ANTS signal at low liposome/AuChi molar ratios (e.g., 1:150 and 1:300) may be attributed to incomplete quenching of ANTS by DPX. The collisional quenching mechanism of this pair of dyes determines that the fluorescence quenching is neither permanent nor complete. These results demonstrate that the absorption of AuChi on liposome surface can effectively prevent them from fusion with one another or filter membranes under rigorous centrifugation and thus significantly improve the stability of the liposomes. These results are also consistent with previous stability study using negatively charged carboxyl-modified gold nanoparticle to stabilize cationic liposomes.[114] As liposome/AuChi molar ratio of 1:300 gave the
most stable formulation, we selected this formulation for the subsequent toxin-triggered drug release studies.

Figure 2.2.3. Fusion ability of AuChi-Liposome at different liposome/AuChi molar ratios. The fluorescent dyes, ANTS and DPX, were encapsulated inside the liposomes at a concentration that DPX maximally quenched the fluorescence of ANTS. Upon fusion with bare liposomes (without AuChi or dyes), the fluorescence of ANTS recovered due to the dilution of the dyes. (A) The measured fluorescence emission spectra of ANTS after incubating ANTS/DPX loaded liposomes in PBS (serving as background fluorescence signal) and in 0.1% Triton X-100 (serving as maximal fluorescence signal), respectively, for 1 h at room temperature. (B) AuChi-Liposome with a liposome/AuChi molar ratio of 1:0, 1:150, or 1:300 were mixed with bare liposomes (without AuChi or dyes) at a molar ratio of 1:4. After incubation for 1 h at room temperature, the bare liposomes were broken by fusing with a centrifugal filter unit. The resulting fluorescence emission intensity of ANTS in the filtrate at 510 nm was measured.
With the liposome:AuChi molar ratio fixed, the liposome formulation was further optimized to obtain the highest pore forming property by bacterial toxin, α-toxin in particular. Alpha-toxin is one of the pore-forming toxins secreted by *S. aureus* bacterium and also the most commonly reported toxin to form pores in artificial or biological membranes.[107] To find an optimal liposome formulation that is the most sensitive to α-toxin, two parameters were investigated; the content of cholesterol in liposome membranes and the addition of polyethylene glycols (PEG) to the liposome solutions. Both parameters have been previously reported to affect the pore-forming activity of toxins in artificial membranes.[117-119] In this study, ANTS/DPX dyes containing liposomes with different cholesterol levels (e.g., 0 wt%, 10 wt%, 25 wt%, and 50 wt%) were prepared and then incubated with α-toxin (20 µg/mL) for 1 h prior to measuring the fluorescence emission of ANTS. Maximal dye leakage was obtained by lysing all liposomes with 1 % (v/v) Triton X-100, while fluorescence emission of the dyes from corresponding liposomes in PBS served as background signal. Percentage of pore forming by α-toxin was calculated using the formula: Percentage of pore forming (%) = (I_{α\text{-toxin}} - I_{PBS})/(I_{TX-100} - I_{PBS}) x 100, in which I_{α\text{-toxin}}, I_{PBS}, and I_{TX-100} represent fluorescence emission intensity at 510 nm of the liposome formulations incubated with α-toxin, PBS, and Triton-X-100, respectively. As shown in Figure 2.2.4A, increase in pore forming was observed when cholesterol content increased, suggesting that cholesterol augments the pore forming efficiency of α-toxin. It was found that 50 wt% of cholesterol in the liposome membrane allowed maximal pore forming activity of α-toxin. It has been
hypothesized that cholesterol can promote the interaction between α-toxin and phosphatidylcholine headgroup[119] or interact with α-toxin itself.[118] Next we fixed the cholesterol concentration at 50 wt% in the liposome formulation and investigated the effects of PEG on the pore forming activity of α-toxin. ANTS/DPX containing liposomes were first mixed with PEG at different PEG concentrations ranging from 0 to 150 mg/mL and then incubated with α-toxin for 1 h, followed by quantifying the percentage of pore forming. As shown in Figure 2.2.4B, when PEG concentration in the solution increased from 0 to 100 mg/mL, pore forming increased and then reached the maximum at 100 mg/mL. However, the pore forming dropped when the PEG concentration was higher than 100 mg/mL. The role of PEG is to dehydrate liposome surfaces because of its strong hydrogen bonding with water, and thus to facilitate the membrane insertion process of toxins.[117] These results suggest that the most sensitive liposome formulation to α-toxin contains 50% cholesterol in the liposome membrane and 100 mg/mL PEG in the solution.
Figure 2.2.4. Toxin-induced pore forming in liposome membranes at various concentrations of cholesterol and PEG. (A) Liposomes with 0, 10, 25, and 50% (w/w) cholesterol were incubated with 20 µg/mL α-toxin for 1 h at room temperature. The dyes released from the pores were quantified by measuring fluorescence emission intensity of ANTS at 510 nm. Percentage of pore forming was obtained by comparing the α-toxin induced dye release with complete dye release caused by 1% (v/v) Triton-X-100. (B) Liposomes with 50% (w/w) cholesterol were incubated with 20 µg/mL α-toxin for 1 h at room temperature in the presence of various concentrations of PEG molecules (M_n=2000 Da), ranging from 0 to 150 mg/mL.
Once the toxins insert into the membrane, the assembled protein oligomers are stable over a wide range of pH and temperature and the formed transmembrane pores stay open at normal conditions. Through these pores, drug payloads can be released from the liposomes. In order to verify our hypothesis of using toxins to form pores and trigger the release of drugs from AuChi-Liposome, we chose MRSA as a bacterium model that secretes toxins and vancomycin as an antibiotic model that has strong inhibitory effects against MRSA bacteria. In the study, optimal formulation of AuChi-Liposome determined from the above studies were loaded with 10 mg/mL of vancomycin and incubated with MRSA252 bacteria (1x10^8 CFU/mL) in 5% tryptic soy broth (TSB) at 37°C. At predetermined time points, released vancomycin was collected from the mixture solution using a centrifugal filter unit with a molecular weight cut-off of 100 KDa. The concentration of vancomycin was determined by reversed phase HPLC. In the experiment, the final vancomycin concentration was about 62 µg/mL. As the minimal inhibitory concentration (MIC) of vancomycin against MRSA bacteria is about 2 µg/mL,[120] we hypothesize that the amount of vancomycin absorbed by cell membranes will not significantly affect the measurement of vancomycin release kinetics. In the study, we first measured the UV absorbance intensity at 280 nm of a series of vancomycin samples ranging from 0-100 µg/mL to generate a standard curve (Figure 2.2.5, inset). Then the concentration of the released vancomycin was quantified by comparing the measured absorbance intensity with the standard curve. As shown in Figure 2.2.5, at 0.5 h and 24 h post incubation of vancomycin-loaded AuChi-Liposome with MRSA bacteria, 29.5 µg/mL
and 62.0 µg/mL of vancomycin were detected in the release medium, which translate to accumulative drug release of 48% and 100% of the total encapsulated vancomycin, respectively. In contrast, no free vancomycin was detected at either time point when the vancomycin-loaded AuChi-Liposome were incubated in the absence of MRSA bacteria. This further confirms that AuChi-Liposome remained stable during the centrifugation process and thus the vancomycin detected in the presence of MRSA was solely contributed by the bacterial toxins through forming pores on liposome membranes. Since 24 h are a standard incubation time to study antimicrobial activity of antibiotics, complete drug release from vancomycin-loaded AuChi-Liposome obtained at this time point implies the potential application of this system to efficiently suppress bacterial growth.
Figure 2.2.5. Accumulative vancomycin release profile from vacomycin-loaded AuChi-Liposome after incubation with MRSA bacteria (1x10^8 CFU/mL) for 0.5 h and 24 h, respectively. The released vancomycin was quantified by reversed phase HPLC. The corresponding samples incubated with PBS (without MRSA bacteria) were used as negative controls. Inset: the linear calibration standard curve of vancomycin at various concentrations measured by HPLC.

After having demonstrated the drug release from AuChi-Liposome in the presence of toxins secreted by MRSA bacteria, we further examined the ability of vancomycin-loaded AuChi-Liposome to inhibit the growth of MRSA252 in vitro. Vancomycin-loaded AuChi-Liposome were incubated with MRSA252 (1x10^8 CFU/mL) in 5% TSB for 24 h, followed by OD_{600} measurement to determine the bacterial growth. Vancomycin-loaded liposomes without AuChi stabilization and free vancomycin were used as positive controls; blank AuChi-Liposome (without vancomycin) and PBS served as negative controls. As shown in Figure 2.2.6,
vancomycin AuChi-Liposome were able to inhibit the growth of MRSA252 to the same extent as vancomycin liposomes and free vancomycin. The student t-test showed that the difference between the OD$_{600}$ value of vancomycin AuChi-liposome and that of vancomycin was insignificant with a $p$-value of 0.18 ($p>0.1$). The obtained OD$_{600}$ signal of vancomycin AuChi-liposome has subtracted that of AuChi-liposome (without vancomycin) to exclude any possible interference signal from the bare liposomal drug carriers. The observed non-negligible inhibitory effects of AuChi-liposome in Figure 2.2.6 might be due to some intrinsic properties of lipids and/or the interactions between unbound AuChi nanoparticles and the bacteria. Although both vancomycin AuChi-Liposome and vancomycin liposome inhibited the growth of MRSA252 bacteria, their working mechanisms were different. Vancomycin AuChi-Liposome were stabilized against fusion and did not release drugs in the absence of bacterial toxins. Thus, their observed inhibitory effect was merely due to the released vancomycin through the pores formed by bacterial toxins. In contrast, vancomycin liposome was not protected by AuChi and could readily fuse with each other and bacterial membranes resulting in vancomycin release, which answered for the observed inhibitory effect. Comparing to bare vancomycin liposome, the vancomycin AuChi-Liposome system exhibits several distinct advantages. First, it improves the shelf-time of the liposome formulation that minimal amount of drugs will be released prior to administration. Secondly, it enables bacteria-targeted antibiotic delivery. As this formulation doesn’t fuse with biological membranes, the drugs will only be released at the infectious sites where the bacteria secrete toxins. Lastly, the dosage of the
antibiotics is self-regulated by the severeness of the infections. More bacteria will secrete more toxins and thus trigger more drug release. Note that the minimum inhibitory concentration (MIC) of vancomycin against MRSA is about 2 µg/mL.[120] The released vancomycin from vancomycin AuChi-Liposome had a concentration up to 62 µg/mL, which should be sufficient to inhibit the growth of the bacteria.

**Figure 2.2.6.** Antimicrobial activity of vancomycin AuChi-Liposome against MRSA bacteria. Vancomycin AuChi-Liposome were incubated with MRSA bacteria (1x10^8 CFU/mL) in 5% TSB for 24 h in the presence of 100 mg/mL PEG. The toxins secreted by the bacteria form pores in the AuChi-Liposome and release the encapsulated vancomycin, which subsequently inhibits the growth of the bacteria. The bacterial growth rate was determined by measuring absorbance at 600 nm after incubation. Vancomycin liposome (without AuChi) and free vancomycin with the same drug concentration (62 µg/mL) served as positive controls. AuChi-Liposome (without vancomycin) and PBS served as negative controls. Data represent mean ± SD (n=3).
2.2.4 Conclusions

In conclusion, a novel passive targeting antimicrobial drug delivery platform was developed, in which bacterial toxins were utilized to trigger antibiotic release from gold nanoparticle-stabilized liposomes for inhibiting the growth of the toxin-secreting bacteria. We systematically optimized the liposome composition and the coverage of chitosan modified gold nanoparticles on the liposome surface so that the liposome fusion activity and undesirable drug leakage were prohibited at normal storage condition, while the liposomes were still susceptible to pore-forming toxins. Once incubated with toxins, the liposomes became leaky and the encapsulated antibiotic payloads were rapidly released through the toxin-formed pores. We further demonstrated that in the presence of toxin-secreting bacteria, 100% of the encapsulated antibiotics were released from the gold nanoparticle-stabilized liposomes and bacterial growth was effectively inhibited by the released antibiotics in 24 h. This antimicrobial drug delivery approach provides an entirely new paradigm for the treatment of bacterial infections by specifically releasing drugs at the infectious sites while minimizing possible off-target effects. While vancomycin was used as an anti-MRSA antibiotic in this study, this technique can be generalized to deliver a variety of antimicrobials and antibiotics for the treatment of various infections caused by bacteria or other organisms that secrete pore-forming proteins.

Chapter 2, in full, is a reprint of the material as it appears in ACS Nano, 2010, Dissaya Pornpattananangkul, Sage Olson, Santosh Aryal, Marta Sartor, Chun-Ming
Huang, Kenneth Vecchio, and Liangfang Zhang, and, in full, in Journal of the American Chemical Society, 2011, Dissaya Pornpattananangkul, Li Zhang, Sage Olson, Santosh Aryal, Marygorret Obonyo, Kenneth Vecchio, Chun-Ming Huang, and Liangfang Zhang. The dissertation author was the primary investigator and author of these papers.
3.1 Introduction

Acne vulgaris (commonly called acne) is a skin disease that is most common during adolescence, afflicting more than 85% of teenagers and over 40 million people in the United States alone [121, 122]. Acne is inflammatory and associates with the immune response to Propionibacterium acnes (P. acnes), a Gram-positive bacterium that colonizes sebum-rich follicles [123]. The entire genome analysis of P. acnes has revealed numerous genes that regulate products involved in degrading host molecules and triggering inflammation [124]. It has been reported that P. acnes releases
chemoattractants that attract the immune system cells such as neutrophils, monocytes and lymphocytes [125, 126]. Previous studies have also found that P. acnes stimulates the production of pro-inflammatory cytokines such as interleukins-1β, -8, -12, and tumor necrosis factor-α [127]. Besides acne, the overgrowth of P. acnes in human is also associated with many other diseases such as endocarditis and toxic shock syndrome [128].

Antimicrobial agents and antibiotics have been used epicutaneously to treat acne for several decades and are still widely prescribed for acne patients. The oxidizing agent benzoyl peroxide (BPO) has been one of the most frequently used epicutaneous medications to decrease P. acnes population in patients suffering from mild to moderate acne [129]. However, several side effects of BPO have been reported including erythema, scaling, burning, and flare [130]. In contrast, we recently demonstrated that lauric acid (LA), one of the typical free fatty acids found in the human sebum, shows stronger antimicrobial activity than BPO while not inducing any cytotoxicity to human sebocytes [131]. Nevertheless, LA is poorly water soluble, and a solvent such as dimethylsulfoxide (DMSO) is required to dissolve LA into topical dosage forms. DMSO is a penetration enhancer that improves the transport rate through the skin barrier; however, its irritative and toxic side effects have been reported [132]. Furthermore, the conventional dosage forms such as cream, gel, and ointment have some major limitations, for example, they do not penetrate through the pilosebaceous unit efficiently and the effective concentration of drug is not sustained [132].
Liposomes have been extensively studied as a drug carrier since the early 1980s [133]. They have shown great potential to act as a topical delivery system for carrying drugs and skin care products. Liposomes can transport drugs to target sides and maintain a higher drug concentration than conventional dosage forms. As a result, the therapeutic effectiveness of liposomal drugs can be enhanced for several folds [134]. Because of the similarity in lipid composition to the epidermis, liposomes can also enhance dermal and transdermal drug delivery while reducing systemic absorption [135]. The study on liposomes for targeting drugs into the pilosebaceous units has suggested that liposomes are potent drug delivery systems for treating hair follicle-associated disorders such as acne [133]. In fact, Lieb et al. has proved that liposomes delivered much higher drug concentrations to the pilosebaceous unit than conventional drug formulations [136]. During the past few decades, liposomes have been used as carriers to enhance clinical efficacy for a large number of drugs. Pevaryl Lipogel as the first topical liposomal drug in the market was launched in 1988 [133]. In addition, antiacne drug-loaded liposomes such as tretinoin, clindamycin, salicylic acid, and tea tree oil-loaded liposomes have been recently reported [137, 138].

Here we use liposomes to encapsulate LA and deliver it to \textit{P. acnes} without using any solvents such as DMSO. LA is an amphiphilic molecule consisting of a hydrophobic hydrocarbon chain and a hydrophilic carboxylic acid headgroup. This structure makes it a good candidate drug to be incorporated into the bilayered wall of liposomes that provides an amphiphilic environment. The present study focuses on
the preparation, characterization, antimicrobial activity, and drug delivery mechanism of LA-loaded liposomes (LipoLA) against *P. acnes* bacteria.

Moreover, to further improve the system and overcome the problem of liposomal instability, the acid-responsive LipoLA system was formulated based on the fact that the acne lesions on human skin are typically acidic with a pH value lower than 5. To provide liposome stability, anionic carboxyl-modified gold nanoparticles (AuC) are allowed to adsorb onto the surface of anionic LipoLA that are preincubated cationic bivalent Mg$^{2+}$ ion (Figure 3.1). This cation mediates attraction of AuC to anionic liposomes via electrostatic bridging interaction. The bound gold nanoparticles effectively prevent liposomes from fusing with one another at neutral pH value, while at acidic acne lesions (e.g. pH<5), the gold particles fall off from liposome surface, allowing liposomes to fuse with the cell membrane of *P. acnes* and thus efficiently deliver lauric acid to kill the bacteria.
Figure 3.1. Schematic illustrations of carboxyl modified gold nanoparticles (AuC)-stabilized lauric acid loaded liposome and its destabilization at acidic pH. The binding between two anionic particles mediates by bivalent cation (Mg\(^{2+}\)). At neutral pH, the liposome is stabilized by deprotonated AuC (Au-COO\(^-\)). The bound gold particles Au-COO\(^-\) are protonated to form Au-COOH and detach from the liposomes when the environment acidity increases to pH<5, resulting in the formation of bare liposomes that can actively fuse with various biological membranes.

### 3.2 Experimental Methods

#### 3.2.1 Materials

Hydrogenated L-a-Phosphatidylcholine (Egg PC), cholesterol, C6-NBD Phytosphing, and 1,2-Dimyristoyl-sn-Glycero-3-Phosphoethanolamine-N-Lissamine Rhodamine B Sulfonyl (DMPE-RhB) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). Lauric acic (LA), hydroxyethyl cellulose (HEC), glycerin, and 18-crown-6 were obtained from Sigma Aldrich (St Louis, MO). Polyethylene glycol 400
(PEG 400) was purchased from USB Corporation (Cleveland, OH). KHCO₃ was from Fisher Scientific (Pittsburgh, PA). 3,4-difluorophenacyl bromide was purchased from Maybridge (Cambridge, UK). Brucella broth, Gas-Pak, and agar were purchased from BD (Sparks, MD). Defibrinated sheep blood and hemin and vitamin K solutions were from Remel (Lenexa, KS). Reinforced clostridium medium was from Oxoid (Hampshire, UK). Institute of Cancer Research (ICR) mice were purchased from Charles River (Wilmington, MA). Commercial Nair® cream, 10% Benzoyl Peroxide (Clean & Clear®), and 2% Salicylic Acid (Clean & Clear®) were purchased from a local drug store. In order to prepare carboxyl functionalized gold nanoparticles (AuC), the following chemicals were purchased: hydrogen tetrachloroaurate (HAuCl₄) (ACROS Organics), Sodium borohydride (NaBH₄) (ACROS Organics), and 3-Mercaptopropionic acid (MPA) (Sigma Aldrich). Potassium hydrogen phthalate and potassium phosphate monobasic were purchased from EMD and Sigma Aldrich, respectively, in order to prepare buffer solutions.

3.2.2. Preparation and characterization of LA-loaded liposomes (LipoLA) and acid-responsive LipoLA

Large unilamellar LA-loaded liposomes (LipoLA) were prepared by the well-known vesicle extrusion technique [139]. Briefly, liposomes composed of 4.5 mg of Egg PC, cholesterol and LA (9:1:0, 8.5:1:0.5, 8:1:1, 7:1:2 and 5:1:4, weight ratio, respectively) were dissolved in 3 mL of chloroform, which was then evaporated by
leading argon gas for 15 minutes. The resultant lipid films were then stored over night under high vacuum to remove any traces of chloroform. The dried lipid films were then rehydrated with 3 mL of deionized water or sterile PBS buffer (1X, pH = 7.4). The suspensions of lipid were vortexed for 5 minutes and then sonicated for 10 minutes in a bath sonicator (Fisher Scientific FS30D) to produce multilamellar vesicles (MLVs). A Ti-probe (Branson 450 sonifier) was used to sonicate the MLVs for 1-2 minutes at 20 W to produce small unilamellar vesicles (SUVs). The resulting SUVs were then extruded through a 100 nm pore-sized polycarbonate membrane for 11 times to form the final products of LipoLA.

To Prepare LipoLA cellulose gel, HEC, Glycerin, and PEG 400 (7.0, 5.6, and 1.8 w/w %) were first swelled by 50 v/v % of PBS under stirring for 15 min on 60°C hot plate. Gel was then left to continue stirring for 24 hours at room temperature. The pre-swelled gel was then mixed with a 50 v/v % LipoLA solution to obtain LipoLA cellulose gel and vortexed until completely homogenized.

Acid-responsive LipoLA was prepared by mixing AuC (1.70 \( \mu \)M), MgSO\(_4\) solution (17.0 \( \mu \)M), and LipoLA (430 \( \mu \)g/mL), following with 10-min sonication.

The size and zeta potential of the LipoLA were measured using the Malvern Zetasizer ZS (Malvern Instruments, UK). The mean diameters of LipoLA were determined through dynamic light scattering (DLS) and the zeta potential was determined from electrophoretic mobility measurements. All characterization measurements were repeated three times at 25°C.
3.2.3. LA loading yield efficiency studies

After preparation, LipoLA, which has an initial LA input of 0, 25, 50, 100, and 200 µg/mL, respectively, was dried by rotavapor (Buchi, Model R-124, Switzerland). Subsequently, the samples were dissolved in methanol and derivatized with phenacyl ester following a published protocol [140]. Briefly, the samples (10 µL) were mixed with 10 µL of KHCO₃ (40 mM in aqueous solution). The solutions were then mixed with Vortex mixer and dried overnight in a vacuum oven (Isoemp model 280A). Consequently, the samples were mixed with 20 µL of Phenacyl-8 (a solution of 0.1 M phenacyl bromide and 0.005 M 18-crown-6 in acetonitrile) and agitated at 80°C for 30 min using a thermomixer (Eppendorf). Samples (20 µL) were mixed with a solution (220 µL) composing of 2 mL of 0.1 M trifluoroacetic acid and 8 mL of acetonitrile. The resulting solutions were assayed by reversed phase HPLC using Agilent 1100 series LC-MSD-Trap-SL system with an electrospray ionization source equipped with a low-flow nebulizer in positive mode (Rev B.01.03, Agilent technologies). Samples were injected into a Discovery HS C₁₈ column (3 µm, 2.1 mm ID x 5 cm, Sigma Aldrich) with an injection volume 25 µL. The mobile phase composed of methanol and water. The concentration gradient was 80:20 v/v at the beginning, 90:10 v/v at 10 min, and 80:20 v/v at 20 min. The flow rate was 0.2 mL/min. Derivatized LA was detected by UV/VIS detector at 254 nm, and the detector temperature was 20 °C.
3.2.4. Preparation of bacteria

*P. acnes* (ATCC 6919) (American Type Culture Collection, Manassas, VA) was cultured on Brucella broth, supplemented with 5\% (v/v) defibrinated sheep blood, vitamin K (5 µg/mL), and hemin (50 µg/mL), under anaerobic conditions using Gas-Pak at 37°C. Single colonies were inoculated in reinforced clostridium and cultured at 37°C until reaching around OD\textsubscript{600}=1.0 (logarithmic growth phase) under anaerobic conditions. The bacteria were harvested by centrifugation at 5,000 x g for 10 min, washed with PBS, and suspended to appropriate amount of PBS for the experiments.

3.2.5. Antimicrobial activity of LipoLA and acid-responsive LipoLA

To determine the antimicrobial activity of LipoLA, two sets or samples were investigated. (i) LipoLA with a constant LipoLA concentration and a LA loading concentration of 0, 12, 33, 80, and 102 µg/mL, respectively. In this set, the LipoLA concentration of each sample was constant while the LA concentration per liposome was different. (B) LipoLA with a LA loading concentration of 25.5, 51, and 102 µg/mL, respectively. However, the 25.5 µg/mL LipoLA and the 51 µg/mL LipoLA were obtained by diluting the 102 µg/mL LipoLA solution with PBS fourfold and twofold, respectively. In this set, the LipoLA concentration of each sample was different while the LA concentration per liposome was constant. PBS (1X, pH=7.4) and empty liposomes (without LA) were used as negative controls. All samples were
incubated with *P. acnes* (1x10^7 CFU/mL) at 37°C for 5 hours under anaerobic condition. After incubation, the samples were diluted 1:10 to 1:10^6 in PBS, and 5 μL of dilutions was spotted on Brucella Broth agar plates supplemented with 5% defibrinated sheep blood and hemin and vitamin K. Agar plates were incubated at 37°C under anaerobic condition for 3 days, and CFU (colony forming units) of *P. acnes* was quantified.

Antibacterial activity of acid-responsive LipoLA was determined by adjusting pH of system to pH 4 to 7 using 5 mM potassium hydrogen phthalate or potassium phosphate monobasic buffer prior to incubation with *P. acnes*. The samples were incubated for 5, 7.5, and 10 min before dilution and spotting on agar plates. Buffer 4 and acid-responsive bare liposome (EggPC/Cholesterol = 6/4 by weight) were used as negative controls.

### 3.2.6. Bacteria fusion studies

Fürster resonance energy transfer (FRET) [94] was preformed to investigate the interaction mechanism between LipoLA and *P. acnes*. To prepare a FRET-pair labeled LipoLA, a fluorescent donor (C₆NBD, 0.1 mol %) and a fluorescent quencher (DMPE-RhB, 0.5 mol %) were simultaneously incorporated into the LipoLA (102 μg/mL LA) by mixing the donor and acceptor with eggPC, cholesterol, and LA before the formation of LipoLA. The resulting LipoLA was then diluted with PBS twofold to prepare LipoLA (51 μg/mL LA). Subsequently, the diluted LipoLA
solutions were mixed with *P. acnes* (8x10⁷, 1.2x10⁸, 1.6x10⁸, and 3.2x10⁸ CFU/mL, respectively). The total volume of the final solution (LipoLA + *P. acnes*) was 1 mL. After 10 min incubation at room temperature, samples were centrifuged at 13,500 rpm for 1 min to remove the excess amount of LipoLA and were resuspended in PBS (1 mL). Consequently, emission spectra in the region of 500-700 nm were obtained by exciting the sample at 470 nm using fluorescent spectrophotometer (Infinite M200, TECAN, Switzerland). Solution of LipoLA (51 µg/mL LA) without incubating with *P. acnes* was used as a negative control. Fluorescence emission of each sample was subtracted with background signal, which was the emission of *P. acnes* itself with the corresponding concentration.

In case of acid-responsive LipoLA, LipoLA was labeled with 0.5 mol % of DMPE-RhB prior to gold-liposome complex formation. The pH of acid-responsive LipoLA was adjusted from 4 to 7 using 5 mM buffer solutions, incubated for 10 min, and centrifuged for 6 min at 13.2 krpm. Then, the supernatant was collected and incubated with *P. acnes* (5 x 10⁸ CFU/mL) for 15 min, following with 2 min centrifugation at 13.2 krpm to collect *P. acnes* pellet. Fluorescent signal of *P. acnes* pellet was then recorded by exciting the sample at 500 nm and collecting emission signal at 580 nm.
3.2.7. Antimicrobial activity of LipoLA *in vivo*-intradermal injection model

Effect of LipoLA on *P. acnes* was determined in a physiological environment using ICR mouse ears. Right before injection, LipoLA (2, 4, 6, and 8 mg/mL) was mixed with *P. acnes* (10^8 CFU/mL). The resulting solution (10 µL) was intradermally injected into the ear of mice. *P. acnes* in benzoyl peroxide (16 mg/mL) in 5% DMSO and PBS served as a positive and negative control respectively. The ears were collected 24 hrs after injection, using a 8 mm biopsy punch and homogenized in 1 mL of sterile PBS with homogenizer (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 (colony forming units) 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. The study was performed with six separate experiments.

3.2.8. Antimicrobial activity of LipoLA *in vivo*-topical model

In this model, the surface of the mouse ear was first scratched with a 25G needle tip to generate a small wound with an area of around 10 mm^2. Then, 1 µL of *P. acnes* (10^9 CFU/mL) was inoculated on the wound to yield 10^6 CFU of *P. acnes* per ear. After 10 min of inoculation, LipoLA gel was applied topically onto the wound. The drug was applied daily for 2 days successively. Commercial BPO cream and PBS gel were applied as positive and negative controls, respectively. After 2 days of drug
application, the ears were collected, and the same homogenization procedure as above was performed. The study was performed with six separate experiments.

### 3.2.9. Skin toxicity

The toxicity of LipoLA was studied on both the back and the ear of ICR mice. For the study done on the back, the back was shaved 24 h prior to the study. Then, LipoLA gel (2 mg/mL) was applied. PBS gel served as a negative control. Commercial Nair® cream, 10% Benzoyl Peroxide cream, and 2% Salicylic Acid gel were used as positive controls. After a 24 hour-period and a 7 day-period, the skin morphology was observed and imaged. Skin irritation was scored according to Draize’s system [141]. For histological observation, the skin of the back was cross-sectioned by an 8 mm biopsy punch, stained with hematoxylin and eosin (H&E), and viewed under a microscope. The experiment on the ear was performed without shaving. After drug exposure, the ear was H&E stained and observed under a microscope. This study was performed with n = 3.

### 3.3 Results and Discussion

Liposome size is an important parameter in using liposomes as drug carriers in regards to encapsulation and adsorption efficiency [142]. *P. acnes* is commonly measured to be 0.3-1.3 µm in diameter and 1-10 µm in length [143]. For notable
antimicrobial activity, it may be essential to construct small liposomes to maximize the amount of liposome uptake by *P. acnes* (i.e. large accumulation LA into *P. acnes*). In addition, small liposomes (diameter, 30~200 nm) are usually unstable; they are prone to fuse with cell membranes, bacterium membranes or other artificial membranes to reduce their high surface tension. It has also been demonstrated that smaller liposomes with diameters near 100 nm have improved penetration through skin compared to larger ones [133]. So LipoLA with a diameter of ~100 nm were prepared for the experiments to evaluate their potential antiacne activity. The vesicle size of LipoLA was characterized through two parameters: z-average mean size and polydispersity index, both of which were calculated from cumulant analysis of dynamics light scattering measurements. As shown in Figure 3.2, the z-average mean size of LipoLA was in the range of 113.6 ± 1.6 nm and 123.6 ± 1.5 nm when the initial LA input varied from 0~200 µg/mL. These results suggest that the increase of LA concentration has no significant effect on the liposome size. We also observed that LipoLA prepared by vesicle extrusion technique has monomodal size distribution with a polysidersity index less than 0.15, which is the characteristic of relatively narrow distribution of particle sizes.

Liposome surface charge is another important factor to determine the interaction of liposomes with biological substances, and it can be characterized by the surface zeta-potential. Binding or uptake of a compound to the phospholipid membrane of a liposome can cause changes in the electrostatic potential profile across the membrane and surface, and the changes can be evaluated through the
measurement of zeta potential [144]. The zeta potential of LipoLA in deionized water determined by electrophoretic mobility measurements are summarized in Figure 3.2. The zeta-potentials of LipoLA formulated with 0, 25, 50, 100, and 200 µg/mL initial LA input were -8.4 ± 0.1, -23.3 ± 0.9, -29.3 ± 1.2, -37.7 ± 0.4 and -51.1 ±3.3 mV, respectively. These results showed that the liposomes with and without LA were negatively charged and an increase of LA concentration led to a decrease of the zeta-potential. Comparable results were obtained for the corresponding LipoLA formulations in PBS buffer (pH = 7.4). Due to the high ionic strength of the media, the variation of zeta-potential of different LipoLAs was significantly reduced. Briefly, the zeta-potentials of LipoLA formulated with 0, 25, 50, 100, and 200 µg/mL initial LA input were -3.1 ± 0.4, -6.9 ± 0.4, -10.1 ± 0.7, -14.6 ± 1.6 and -22.5 ± 2.1 mV, respectively. Free fatty-acids have a pK value of approximately 5, thus at near physiological pH of 7.4 the carboxyl group of the free fatty acid will deprotonate and attribute to the negatively charged surface of the liposome. This explains the observation of an increase of LA concentration in liposome leading to a decrease of the zeta-potential, and confirms that LA is indeed incorporated into the liposomes.
Figure 3.2. Characterization of lauric acid-loaded liposomes (LipoLA). LA at various concentrations ranging from 0~200 µg/mL was mixed with other lipid components to prepare LipoLA. The size (diameter, nm) and surface zeta potential (mV) of the LipoLA were determined by dynamic light scattering.

The loading yield of LA in LipoLA formulations was evaluated by HPLC. When using the HPLC technique to separate LA from the other components in LipoLA, LA must be derivatized with phenacyl ester first because the functional group of LA is a carboxylic group which has negligible UV absorbance. After being derivatized with phenacyl bromide, derivatized LA could be detected by the UV/VIS detector at 254 nm. We first measured the UV absorbance intensity of a series of derivatized LA samples ranging from 0-1500 µg/mL to generate a standard curve (Figure 3.3A). Then the loading concentration of LA in all LipoLA formulations was quantified by comparing with the standard curve. As shown in Figure 3.3B, for the LipoLA formulations with an initial LA input concentration of 0, 25, 50, 100, and 200
µg/mL, their LA loading yield was 0, 12, 33, 80, and 102 µg/mL, respectively. The corresponding drug encapsulation efficiency was 50%, 66%, 80%, and 51% respectively. These results indicated that the encapsulation efficiency of LA increased as the weight ratio of LA increased until it reached 100 µg/mL of the initial LA input. At 200 µg/mL of the initial LA input, however, encapsulation efficiency dropped. This might be because the LA loading reaches a saturation point at the range of 100-200 µg/mL initial input. These results also indicated that some LA was lost during the LipoLA preparation steps which could be extrusion and filtration steps. Using needle sonicator, the samples were in a close contact with the needle, so some LA might adhere to the needle. In addition, LA may stick with extrusion membrane or apparatus itself resulting in the lost of LA.
Figure 3.3. Quantification of LA loading in LipoLA by HPLC. (A) UV absorption intensity of derivatized LA at the concentrations of a-f: 50, 100, 200, 500, 1000, and 1500 µg/mL. Inset: the corresponding linear calibration standard curve. (B) The loading of LA in the LipoLA formulations with an initial LA input of 0, 25, 50, 100, and 200 µg/mL was 0, 12, 33, 80, and 102 µg/mL, respectively. Data represent mean ± SD of three individual experiments.
Five samples of LipoLA (0, 12, 33, 80, and 102 µg/mL LA) were incubated with *P. acnes* (1x10^7 CFU/mL) for 5 hours under anaerobic condition in order to determine their antimicrobial activity. The result showed that LipoLA with 102 µg/mL LA completely killed the bacteria (Figure 3.4A). In these five samples, the LA loading concentration was different but the molar concentration of liposome in each sample was kept constant. To further investigate the system, LipoLA with 102 µg/mL LA was diluted twofold and fourfold to obtain 51 and 25.5 µg/mL LipoLA, respectively, and their antimicrobial activities were determined. Surprisingly, experiment results showed that *P. acnes* was completely killed by 51 µg/mL LipoLA (Figure 3.4B). One possible way to explain this phenomenon is the following reasons. By diluting 102 µg/mL LipoLA to 51 µg/mL LipoLA, the amounts of LA encapsulated inside each liposome of these two samples were identical although the amount of liposome per volume of 51 µg/mL LipoLA was lower. Due to the small size of *P. acnes* (0.3-1.3 µm in diameter and 1-10 µm in length), only a few LipoLA (~120 nm) could interact with each *P. acnes* bacterium, and for 51 µg/mL sample, the amount of liposome was enough to interact with all bacteria in the solution, thus the fully killing effect was observed. On the other hand, full killing effect of 80 µg/mL LipoLA in the previous set of experiments (Figure 3.4A) was not observed because the amount of LA in each liposome was not high enough to kill bacteria even though the amount of liposome of was enough to interact with all of bacteria. From these results, it was obvious that antimicrobial activity of LipoLA was determined mostly by the amount of LA loaded in each liposome, not the number of liposome per
volume solution because one colony of *P. acnes* could interact only with a few liposomes.

**Figure 3.4.** Antimicrobial activity of LipoLA against *P. acnes*. Two sets of LipoLA were incubated with *P. acnes* (1x10^7 CFU/mL), respectively, for 5 hours under anaerobic conditions to test their antimicrobial activity. (A) LipoLA with a LA loading concentration of 0, 12, 33, 80, and 102 µg/mL, respectively. In this set, the LipoLA concentration of each sample was constant while the LA concentration per liposome was different. The results showed that 102 µg/mL LipoLA completely killed *P. acnes*. (B) LipoLA with a LA loading concentration of 25.5, 51, and 102 µg/mL, respectively. However, the 25.5 µg/mL LipoLA and the 51 µg/mL LipoLA were obtained by diluting the 102 µg/mL LipoLA solution with PBS fourfold and twofold, respectively. In this set, the LipoLA concentration of each sample was different while the LA concentration per liposome was constant. The results showed that both 51 µg/mL LipoLA and 102 µg/mL LipoLA completely killed bacteria. Incubation with PBS buffer and empty liposome solution (without LA) served as negative controls. Data represent mean ± SD of three individual experiments. UD: undetectable.
In order to further understand the interaction mechanism between LipoLA and the bacteria, we labeled LipoLA with a FRET pair of chromophores and monitored the FRET signal changes upon mixing LipoLA with the bacteria at various conditions. FRET is a widely used technique that precisely measures the distance of two subjects at the molecular level based on an energy transfer mechanism of two chromophores [94, 145]. Typically, the two subjects are labeled with a donor chromophore and an acceptor chromophore, respectively. The donor chromophore in its excited state can transfer energy to the acceptor chromophore in close proximity (<10 nm) through a nonradiative long-range dipole-dipole coupling mechanism. Here we incorporated a fluorescence donor (C\textsubscript{6}NBD: excitation/emission= 470 nm/520 nm) and a fluorescence acceptor (DMPE-RhB: excitation/emission= 550 nm/580 nm) into the lipid membranes of LipoLA. By controlling the molar ratio between the donor and the acceptor, we prepared the LipoLA in which the fluorescence emission from the donor was completely quenched by the acceptor. We hypothesized that if the LipoLA fuses with bacterial membranes, the spread of the donor and acceptor chromophores within the bacterial membranes will alleviate or eliminate the FRET efficiency, resulting in fluorescence recovery of the donor. To this end, we mixed the FRET-pair labeled LipoLA with several concentrations of \textit{P. acnes}, followed by removing the excess LipoLA. The samples were then excited at the wavelength of 470 nm. As shown in Figure 3.5, the rise in the emission peak of C\textsubscript{6}NBD at 520 nm was detected when the concentration of \textit{P. acnes} increased indicating spatial separation between C\textsubscript{6}NBD and DMPE-RhB increased and the efficiency of energy
transfer decreased. The changes of the emission peaks of DMPE-RhB, however, were neglected in our studies for the reason that the DMPE-RhB could be excited by not only the FRET from C6NBD but also excitation wavelength at 470 nm. This made it difficult to make a conclusion based on the emission changes of DMPE-RhB. LipoLA at the same concentration while not mixing with bacteria was used as a control for this study. When the control sample was excited at 470 nm, no emission at 520 nm was detected but the emission peak at 580 nm was observed.

These observations indicate that the interaction mechanism between LipoLA and *P. acnes* was fusion; not adsorption or aggregation. That is, LipoLA fused with the membranes of *P. acnes* and released the carried LA into the bacterial membranes. This finding together with the antimicrobial activity of LipoLA is consistent with the mechanism of LA killing Gram-positive bacteria by separating the inner and outer membranes of the bacteria. This fusion mechanism is also consistent with previous reports of liposomal drug delivery to gram-positive bacteria such as *Staphylococcus aureus* and *Stenotrophomonas maltophilia* [146].
Figure 3.5. FRET measurements of the fusion between LipoLA and *P. acnes*. A fluorescent donor (C₆NBD) and a fluorescent quencher (DMPE-RhB) were simultaneously incorporated into the LipoLA (51 µg/mL LA) with a proper molar ratio that the quencher effectively quenched the fluorescence emission from the donor. The FRET-pair labeled LipoLA was incubated with *P. acnes* at a concentration of a-d: 8x10⁷, 1.2x10⁸, 1.6x10⁸, and 3.2x10⁸ CFU/mL for 10 minutes. After removing the excess LipoLA, all samples were excited at 470 nm. A rise in emission intensity of C₆NBD (donor) at 520 nm was observed with the increasing bacterial concentration, indicating the occurrence of fusion between LipoLA and bacteria that caused the spatial separation of C₆NBD and DMPE-RhB.

After demonstrating the efficacy of LipoLA *in vitro*, we further study the efficacy of our liposomal formulation *in vivo* by intradermal injection into ICR mouse ear. The ear was selected for this study because of its confined structure in order to ensure that all of the inoculated bacteria will remain inside of the injection area. LipoLA (2, 4, 6, and 8 mg/mL) was mixed with *P. acnes* (10⁸ CFU/mL) right before the injection to confirm that the interaction had mostly happened in a physiological environment. Subsequently, the mixture was intradermally injected into the mouse ear. The injection volume used was 10 µL, which is the maximum volume that an ear
can hold without any undesired leakage. It should be noted here that a single injection of mixture was performed instead of a double injection of *P. acnes* and LipoLA separately to prevent the formation of two holes on one ear which will lead to an uncontrollable outflow of sample through the first hole. After 24 hours of injection, the ear was collected using an 8-mm biopsy punch and homogenized to quantify the remaining amount of *P. acnes*. We used 16 mg/mL BPO in 5% DMSO premixed with *P. acnes* as a positive control. *P. acnes* in PBS was used as a negative control, while an injection of PBS without *P. acnes* was used to demonstrate that there was no contamination from other bacteria during the procedure. As shown in Figure 3.6, the number of *P. acnes* detected after the treatment was 3.00, 1.65, 0.56, and 0 log(CFU) for 2, 4, 6, and 8 mg/mL LipoLA respectively. Comparing to the bacteria number of the untreated sample of 4.22 log(CFU/mL), there was a significant reduction in number of *P. acnes* when treated with LipoLA with a correlation to the drug concentration. At the lowest concentration, 2 mg/mL, *P. acnes* number was reduced by more than one order of magnitude, and more reduction was observed when the concentration of LipoLA was increased. At the highest concentration of 8 mg/mL, LipoLA completely eliminated the bacteria. Considering to the positive control, 16 mg/mL of BPO in 5% DMSO, there was a reduction in number of bacteria from 4.22 to 3.20, which was only about one order of magnitude reduction. BPO was selected as a positive control because it is a wildly used OTC acne treatment. It is commonly prepared in many dosage forms, including cream, gel, or lotion, all of which are easy and convenient to apply, but are not suitable for intradermal injection. Thus, in this
study, a solution of BPO was prepared in 5% DMSO, which is the condition that does not generate acute toxicity to human sebocytes or *P. acnes* [147] However, the full effectiveness of this injectable BPO formulation might be impeded here due to BPO’s limited solubility. This could be further improved by using a different dosage form with higher drug solubility and concentration. Moreover, the volume of drug injected here (10 uL) was limited by the ear structure and led to an insufficient amount of administered drug to interact with bacteria, resulting in a requirement of high concentration of LipoLA. Due to these two main reasons, a topical model was developed to better investigate the efficacy of both LipoLA and BPO cream obtained from the local drug store in the subsequent study.
A topical model was developed to overcome some limitations of the injection model as well as to mimic *P. acnes* induced inflamed acne. In the case of inflammatory lesion in human, chemotactic factors are secreted from *P. acnes*, resulting in an attraction of inflammatory cells into the sebaceous follicle. These cells will further release other inflammatory factors, such as lysosomal enzymes, proteases, and reactive oxygen that damage follicle wall, leading to inflammatory lesions [148]. From our previous study using the ICR mouse ear model, it was observed that by inoculating *P. acnes* into the ear, macrophages were attracted into the injection site, similar to *P. acnes* induced inflammatory acne lesions in human [147]. In this study,
$10^6$ CFU of \textit{P. acnes} was inoculated on the ear of the mouse that has pre-scratched wound with an area of around 10 mm$^2$ to generate \textit{P. acnes} inducted inflammation. Following inoculation, 2 mg/mL of LipoLA prepared in gel was applied daily for 2 days. The gel formulation used in this study composed of hydroxyethyl cellulose (HEC), glycerin, and polyethylene glycol 400 (PEG 400) (7.0, 5.6, and 1.8 w%). HEC is a cellulose-derived thickening agent, while glycerin and polyethylene glycol 400 (PEG 400) are emollient. By preparing LipoLA in gel, we have observed a well-preserved antimicrobial activity of liposome from both \textit{in vitro} and \textit{ex vivo} study (data not shown). As shown in Figure 3.7, a 2 mg/mL concentration of LipoLA gel completely killed the bacteria. Considering a positive control, 10% BPO cream that was purchased from a local drug store also expressed complete bacteria elimination. The negative controls of this experiment were non-treatment, which PBS gel was applied and sample without \textit{P. acnes} inoculation. For non-treatment sample, 4.02 log(CFU) of \textit{P. acnes} was detected. The reduction of bacteria number from the initial amount of 6 to 4.02 log(CFU) could be resulted from a clearance from the host’s immune system or some loss during the tissue grinding process, however this recovery number was always consistent with every experiment that was conducted. For samples without \textit{P. acnes}, no bacteria were detected in the experiment, indicating a clean system, free from contaminations from normal flora. This result suggests that LipoLA is as effective as commercialized BPO cream, which has been considered as the gold standard to treat mild to moderate acne due to its effective antimicrobial and oxidizing activity since the 1960s [149]. However, due to BPO’s common side
effects, such as dryness, irritation, and burning, this has resulted in lowered patient compliance and unsuccessful treatment. LipoLA, on the other hand, has shown its equivalent in efficacy, while possibly reducing toxicity significantly. To prove this hypothesis, the toxicity profile of LipoLA was performed \textit{in vivo}, in comparison to BPO, which served as a positive control.

\begin{figure}
\centering
\includegraphics[width=0.5\textwidth]{figure3.7}
\caption{\textit{In vivo} antimicrobial activity of LipoLA investigated by topical model. Wound was generated on mouse ear. Then, \textit{P. acnes} (10$^6$ CFU) was inoculated onto the wound, following with LipoLA gel daily application for 2 days successively. At 72 hours, the ear was collected, and remaining amount of \textit{P. acnes} was quantified. BPO cream from local drug store was a positive control, while PBS gel and none \textit{P. acnes} inoculation were served as negative controls. Data are representative of six separate experiments. UD = undetectable.}
\end{figure}

To study the toxicity profile of LipoLA \textit{in vivo}, the drug was topically applied on the skin of ICR mice, and the change in skin morphology was observed for up to 7 days. In this study, the toxicity of LipoLA was compared with several OTC drugs,
including 10% BPO, 2% Salicylic Acid (SA), and Nair® hair removal cream. SA is a commonly used acne therapeutic due to its keratolytic effect and slight bacteriostatic property [150]. In this study, potential toxicity was observed on both the skin of the back and ears to study the effects on different skin types. For the study done on the backs of mice, the skin was shaved 24 hours prior to the study to allow skin to recover from any possible disturbance to the stratum corneum [141]. Both the area of the back and ears were moistened with PBS prior to exposure to samples. After preparing the skin, the samples were topically applied daily for either 24-hour or 7-day period. Once the allotted time had passed, the drug was removed by Kimwipes that were moistened with PBS and the skin was observed. For the 24 hour study on back (Figure 3.8A), the skin had maintained its normal structure after LipoLA gel contact, and no erythema or edema was observed. This structure was consistent with the PBS gel sample, which served as a negative control. On the other hand, all of OTC drugs, had caused obvious skin damage. Nair® cream was the most irritating chemical, causing severe erythema and moderate edema all over the application area. Both BPO cream and SA gel caused moderate erythema, and appeared as many small red dots on the skin, but no edema was observed. Subsequently, the skin’s reaction was evaluated according to the Draize’s irritation scoring system as shown in Table 3.8B and C. Erythema and edema scores of LipoLA and PBS gel were 0 and 0, indicating no apparent irritation. In the case of Nair® cream, an erythema score of 4 showed severe erythema with beet redness and slight eschar formation, which signified in depth injury. In addition, the average edema score from 3 separate mice
was 1.6, demonstrating slight edema with raised edges of the affected area. For both BPO cream and SA gel samples, the scores were similar, which are 2 and 0 for the erythema and edema scores respectively, representing well defined erythema all over the skin. The back skin histology was further investigated under a microscope. The skin biopsy was collected and stained with hematoxylin and eosin (H&E). An H&E stain is widely used to diagnose various tissue types and morphologic changes. Hematoxylin is blue and stains nucleic acids, while eosin causes a pink color, staining proteins nonspecifically. In H&E stained tissue, nuclei are appeared in blue, and the cytoplasm and extracellular matrix are pink with varying intensity in color [151]. As shown in Figure 3.8D, the histology of the LipoLA treated sample showed an undisturbed structure with a layer of healthy epidermal cells on top of the dermis layer, which was identical to the PBS treated skin sample. On the other hand, the skin structures of positive controls were completely damaged. Nair® cream caused the most severe damage to the structure in which a majority of the defined epidermis layer had disappeared, and many inflammatory cells were accumulated in the dermis layer (arrow head). For both BPO and SA samples, the epidermis layer was destroyed and disconnected from the dermis layer (arrow), and there are significant amount of inflammatory cells in the dermis (arrow head). In addition to these back skin studies, we also tested the toxicity of the sample on the ears. This is because it is easier for the ear area to show a more apparent accumulation of fluid, called edema, which will cause swelling and increased ear thickness because of the thinness of the dermal layer. Furthermore, since the ear is hairless, the possible skin irritation effects from
shaving could be eliminated. Histology images of ears (Figure 3.8E) reaffirmed that edema occurred with all of positive controls, Nair®, BPO, and SA, as the thickness of the skin layer above cartilage increased significantly. Moreover, Nair® induced epidermal destruction (arrow), and an accumulation of inflammatory cells was also found in both Nair® and SA sample (arrow head). These results evidently suggest that within 24 hours, the two most popular OTC acne medications, BPO and SA as well as Nair® hair removal cream significantly induce skin toxicity, while LipoLA does not generate any skin reaction within this time frame.
Figure 3.8. Observation of toxicity of LipoLA on mouse back and ears for 24 hours. LipoLA gel was applied on shaved back and was removed after 24 hours. A) The back was imaged by a camera. B) Draize’s irritation scores indicating erythema. C) Edema score regarding to Draize’s study. D) Back skin was cross-sectioned, stained with haematoxylin and eosin (H&E), and viewed on a microscope. E) The ear was exposed to the drug for the same period of time and H&E stained. PBS gel was used as a negative control, while Nair®, Benzoyl Peroxide (BPO), and Salicylic Acid (SA) were positive controls. Data are representative of three separate experiments with similar results.

Additionally, we further studied the long term effect of LipoLA on the skin by continuously applying the drug on the back and ears daily for 7 days. At the end of study, the drug was removed, and the skin morphology was observed. As shown in Figure 3.9A, Table 3.9B, and 3.9C, LipoLA did not cause any visible irritation on back with observed erythema and edema scores of 0 and 0. The histology of the
dermal area of the back also indicated a healthy skin structure without any inflammation or epidermal damage (Figure 3.9D). In addition, from Figure 3.9E, the undisturbed ear structure further suggests a consistency in no induced toxicity of LipoLA. This result further verifies that LipoLA does not generate any skin irritation for a longer drug application period of up to 7 days, while obvious, visible toxicity is develops in positive controls samples of both skin types within 24 hours. The reason for the non-toxic effect of LipoLA is due to its 100% naturally derived components, as opposed to other widely used acne drugs, which contain many harsh chemicals. In this study, LipoLA was proven to be a safe and natural acne therapeutic based on the ICR mouse model, which signifies that a continuing study in human can be pursued.
Figure 3.9. Observation of toxicity of LipoLA on mouse back and ears for 7 days. LipoLA was applied on shaved back daily for 7 days. A) Digital image of back. B) Draize’s irritation scores indicating erythema and C) edema. D) Cross-sectioned back skin was stained with H&E and investigated by a microscope. E) Ear structure was observed by a microscope. Data are representative of three separate experiments with similar results.

After demonstrating the antimicrobial activity of LipoLA in vitro and in vivo, acid-responsive LipoLA (AuC-Mg-LipoLA) was formulated by mixing LipoLA with MgSO₄ and AuC with a molar ratio of 1:2000:200 under gentle bath sonication for 10 min. Using dynamic light scattering (DLS), the size and surface zeta potential of the formed liposomes were 85.0±1.0 nm and -33.1±1.7 mV, respectively. This vesicle size was within the range of 100 nm which was previously reported to be suitable for
dermal delivery and enhance pharmaceutics penetration into the skin [133]. The acid sensitivity of the system was further evaluated by measuring UV-vis absorption of AuC-Mg-LipoLA at pH=7 and pH=4, respectively, after the removal of unbound AuC by centrifugation. Here pH of the system was adjusted by HCl due to the same reason described in Chapter 2.1. After adjusting pH of the system to 7 and 4, the AuC-Mg-LipoLA was centrifuged to precipitate unbound AuC nanoparticles. The UV-vis absorption spectra of the resulted supernatants were then recorded in the range of 300 nm to 700. As shown in Figure 3.10, supernatant of system at pH 7 strongly showed UV absorption spectrum of AuC; however, at pH 4, the spectrum was not detected, indicating the complete removal of AuC. The observed UV absorption spectra were consistent with the color difference of the supernatant as shown in Figure 3.10 inset. At pH 7, AuC strongly attach to LipoLA, and thus cannot be removed by centrifugal force. However, once the pH was changed to 4, AuC can be removed from the system and formed pellet at the bottom of the tube. This observation indicates that when pH value (e.g. pH=7) is higher than pKa (~5) of carboxylic acid, both of carboxylic group of LA in LipoLA and AuC were in deprotonated form (COO\textsuperscript{-}), thus AuC strongly adsorb onto the surface of LipoLA via electrostatic bridging interaction provided by Mg\textsuperscript{2+}. However, when the pH value was less than the pKa value (e.g. pH=4), carboxylic groups were protonated to COOH form, resulting in a disassembly of the system.
After having demonstrated the binding and detaching of AuC nanoparticles from LipoLA at neutral and acidic pH, the fusion activity of the system with *P. acnes* bacteria from pH 4 to 7 was observed. In this study, LipoLA was prelabeled with RhB fluorescent dye before stabilizing with AuC. The pH of the AuC-Mg-LipoLA was then adjusted from pH=4 to pH=7, respectively, using buffer solutions. The resulting unbound AuC nanoparticles were removed from the solutions via 10 min centrifugation at 1.3x10^4 rpm in order to eliminate fluorescence quenching effect of free AuC in the solutions through random collision. Subsequently, the supernatant was incubated with *P. acnes* for 15 min, and the bacteria pellet was collected and

**Figure 3.10.** UV-vis absorption spectra of AuC-Mg-LipoLA at pH=7 (solid line) and pH=4 (dash line), respectively, after removal of unbound AuC through centrifugation. Inset: AuC-Mg-LipoLA after centrifugation.
suspended in water. The solution was then exited at the wavelength of 500 nm, and the emission signal at 580 nm was collected to quantify fluorescent intensity that was transferred from AuC-Mg-LipoLA to the bacteria. As shown in Figure 3.11, the fluorescent signal dramatically increased when pH is lower than 5, indicating the transfer of RhB dyes from destabilized liposomes to bacterial membrane. On the other hand, at pH 6 and 7, which are higher than pKa of carboxylic group, the fluorescent intensities were relatively low due to the stabilization of liposomes.

**Figure 3.11.** Fluorescent intensity of *P. acnes* bacteria after incubation with RhB-labeled AuC-Mg-LipoLA at different pH. Increased in fluorescent intensity was observed when pH of the system is below 5, indicating a detachment of AuC from RhB-labeled LipoLA and a transfer of RhB dye from liposome to bacterial membrane by fusion.
After having demonstrated the acid responsive property of AuC-Mg-LipoLA system, we further examined the ability of the system to kill *P. acnes* bacteria at acidic condition. The pH of AuC-Mg-LipoLA was adjusted from 4 to 7 prior to incubation with *P. acnes* bacteria for 10 min under anaerobic condition in order to determine its antimicrobial activity. Liposome without LA, but stabilized with AuC (bare lipo) and buffer at pH 4 were selected as negative controls. As shown in Figure 3.12, when pH of the system decreases below 5, the amount of *P. acnes* was decreased, and completely killed at pH 4. However, two controls did not have an effect on the bacteria viability. This result indicates that AuC-Mg-LipoLA system at acidic condition has strong antibacterial property against *P. acnes* due to the delivery of LA, not pH or other factors. This finding is consistent with the fusion study, where AuC-Mg-LipoLA is able to fuse with bacteria only at pH < 5.
Figure 3.12. Antimicrobial activity of AuC-Mg-LipoLA against *P. acnes* from pH 4 to 7. AuC-Mg-LipoLA is able to kill the bacteria when pH is lower than 5. The system without LA (bare lipo) and buffer at pH 4 were served as negative controls. Data represent mean ± SD (n=3).

3.4 Conclusions

In conclusion, lauric acid-loaded liposomes (LipoLA) and acid-responsive LipoLA (AuC-Mg-LipoLA) have been developed to effectively kill *Propionibacterium acnes* (*P. acnes*), the major causing agent of acne vulgaris. In case of LipoLA, the 120-nm particle size and a -43 mV surface charge indicate both a suitable size distribution for skin delivery as well as the full encapsulation of Lauric Acid (LA), a negatively charged antimicrobial free fatty acid within the liposome. It was demonstrated that LipoLA can effectively kill *P. acnes in vitro* by fusing and delivering LA directly to the bacterial membrane, leading to the destruction of the surface structure of the organism. Using the ICR mouse model, we further
demonstrated that LipoLA’s efficacy to eradicate the bacteria is also well preserved *in vivo* from both the intradermal injection and topical administration of drug. In addition, LipoLA generated no skin irritation for up to 7 days, while other OTC acne therapeutics, such as benzoyl peroxide (BPO) and salicylic acid (SA) showed moderate irritation to the skin within 24 hours. The system was further developed by formulating acid-responsive LipoLA. This system was prepared by allowing carboxyl-modified gold nanoparticles (AuC) to adsorb onto the surface of LipoLA via electrostatic bridging interaction of Mg$^{2+}$ ion. It was demonstrated that this system has acid-responsive property, where AuC falls off when pH is lower than the pKa (~5) of carboxylic acid. After its detachment, LipoLA still maintains its fusion property and fuse with bacteria membrane. Lastly, it was observed that AuC-Mg-LipoLA system can completely killed the bacteria at pH 4, but not at pH higher than 5. These findings validate that AuC-Mg-LipoLA has a strong potential to be a stable and effective acne medication.

Chapter 3, in full, is a reprint of the material as it appears in Biomaterials, 2009, Dissaya Pornpattananangkul, Darren Yang, Teru Nakatsuji, Michael Chan, Dennis Carson, Chun-Ming Huang, and Liangfang Zhang, and, in full, in a manuscript submitted to Proceeding of the National Academy of Sciences, 2012, Dissaya Pornpattananangkul, Victoria Fu, Soracha Thamphiwatana, Li Zhang, Chun-Ming Huang, and Liangfang Zhang. The dissertation author was the primary investigator and co-author of theses papers.
Chapter 4 Conclusions and Future Directions

Liposomes have been recognized as one of the most widely used drug delivery systems to treat bacterial infection due to many advantages, including their unique lipid bilayer structure which enables them to readily fuse with microbes, their structure to carry both hydrophilic and hydrophobic molecules, and the ability to attach targeting ligands to their surface in order to selectively bind to microorganisms. However, the applications of liposomes are usually limited by their instability during manufacturing and storage. They are prone to fuse with one another to reduce their surface tension, leading to undesired mixing, payload loss, and an increase in size. These significantly affect their unique property as a nano-scale delivery vehicle, and more importantly, their ability to fuse with targeted bacteria. As a result, we developed liposomes that are stable during storage and able to resume their fusion activity depending on the environmental conditions of target sites,
such as acidic pH and bacterial virulence factor. Moreover, to validate the application of this system to treat bacterial infection, acid-responsive system was selected to evaluate its efficacy against Propionibacterium acnes (P. acnes). The following sections will reiterate the important conclusions from this dissertation in detail.

4.1 Acid-Responsive Liposomes

Liposome system that is stable against fusion at neutral pH (storage condition) and able to resume the fusion activity at acidic environment was developed by attaching carboxyl-modified gold nanoparticles to the outer surface of cationic liposomes. Using extrusion method, the cationic liposomes with consistent size at 90 nm and zeta potential of +25 mV were prepared. After gold nanoparticles adsorption, a slight size increase and the change in surface charge to –25 mV were observed, indicating an adsorption of gold nanoparticle. At neutral pH, the adsorbed gold nanoparticles effectively quenched the fluorescent dyes presented in the lipid membranes, indicating a strong binding of gold nanoparticles. On the other hand, at pH < 5, the fluorescence recovery of the dyes was observed, indicating the detachment of gold nanoparticle from liposome. UV-vis absorbance measurements also confirm the binding and detaching of gold nanoparticles from the liposomes. It was also observed that fusion activity of liposome was prohibited at neutral pH when gold nanoparticles attach to the liposome surface, and the fusion activity resumed at acidic
aevironment. Since the infectious lesions on human skin are typically acidic with a pH<5, these acid-responsive liposomes with tunable fusion ability hold great promise for dermal drug delivery to treat a variety of skin diseases such as acne vulgaris and staph infections.

### 4.2 Virulence Factor-Responsive Liposomes

A new approach to selectively delivering antimicrobials to the sites of bacterial infections by utilizing bacterial toxins to activate drug release from gold nanoparticle-stabilized phospholipid liposomes was demonstrated. The binding of chitosan modified gold nanoparticles to the surface of liposomes can effectively prevent them from fusing with one another and from undesirable payload release in regular storage or physiological environments. However, once these protected liposomes “see” bacteria that secrete toxins, the toxins will insert into the liposome membranes and form pores, through which the encapsulated therapeutic agents are released. The released drugs subsequently impose antimicrobial effects on the toxin-secreting bacteria. Using methicillin-resistant *Staphylococcus aureus* (MRSA) as a model bacterium and vacomycin as a model anti-MRSA antibiotic, we demonstrate that the synthesized gold nanoparticle-stabilized liposomes can completely release the encapsulated vacomycin within 24 h in the presence of MRSA bacteria and lead to inhibition of MRSA growth as effective as an equal amount of vacomycin loaded
liposomes (without nanoparticle stabilizers) and free vacomycin. This bacterial toxin enabled drug release from nanoparticle-stabilized liposomes provides a new, safe and effective approach for the treatment of bacterial infections. This technique can be broadly applied to treat a variety of infections caused by bacteria that secrete pore-forming toxins.

### 4.3 Acid-Responsive Liposomes for the Treatment of Acne Vulgaris

The application of acid-responsive liposomes was demonstrated by testing their efficacy in delivering drug molecules to *Propionibacterium acnes* (*P. acnes*) bacteria, one of the major pathogenic factors of acne vulgaris. In this study, we encapsulated lauric acid (LA), a medium chain free fatty acid with strong antimicrobial activity against *P. acnes*, into the liposome (LipoLA). Because the acne lesions on human skin are typically acidic with a pH value lower than 5, the acid-responsive LipoLA (AuC-Mg-LipoLA) was developed. In this system, negatively charged carboxyl-modified gold nanoparticles (AuC) are allowed to adsorb onto the surface of negatively charged LipoLA with the aid of Mg$^{2+}$, forming an electrostatic bridge between the two nanoparticles. First, we demonstrated the efficacy of LipoLA to effectively kill *P. acnes in vitro* and *in vivo* by fusing and delivering LA directly to the bacterial membrane. *In vivo* toxicity study also indicated that LipoLA did not
generate any skin irritation as other OTC acne therapeutics, such as benzoyl peroxide (BPO) and salicylic acid (SA). In the case of acid-responsive LipoLA, it was demonstrated that the acid-responsive property of this system was preserved. At neutral pH, AuC strongly adsorbed to the liposome’s surface and prevented fusion between these liposomes and *P. acnes*. On the other hand, when pH was decreased to less than 5, AuC detached from the liposome, allowing the liposomes to fuse with the bacteria. Moreover, it was observed that AuC-Mg-LipoLA completely eradicated the bacteria at pH 4, but not at neutral pH. This study demonstrates the application of an acid-responsive system to treat bacteria in acidic environment and clearly indicates its great potential to treat many other skin diseases.
REFERENCES


