8-2022

An Analytical Method to Detect and Quantify LL-37 in an In Vitro Co-culture of Activated THP-1 Cells and Staphylococcus Aureus, Staphylococcus Epidermidis, and Pseudomonas Aeruginosa

Victoria Hunter
University of Arkansas, Fayetteville

An analytical method to detect and quantify LL-37 in an *in vitro* co-culture of activated THP-1 cells and *Staphylococcus aureus*, *Staphylococcus epidermidis,* and *Pseudomonas aeruginosa*

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy in Cell and Molecular Biology

by

Victoria Hunter
University of Arkansas
Bachelor of Science in Biomedical Engineering, 2017

August 2022
University of Arkansas

This dissertation is approved for recommendation to the Graduate Council

Julie A. Stenken, Ph.D.
Dissertation Director

Jeannine M. Durdik, Ph.D.
Committee Member

Ingrid Fritsch, Ph.D.
Committee Member

Jiangchao Zhao, Ph.D.
Committee Member

Abstract

Healthcare-associated infections (HCAIs) affect 1.7 million hospitalized patients each year, resulting in over 98,000 deaths and anywhere from $28.4 to $45 billion in treatment costs. Furthermore, it has been established that more than 80% of these infections are caused by biofilms While HCAIs can be bacterial, viral, fungal, or parasitic, there are between 12 and 17 species cause 80 to 87% of all HCAIs. Among the most commonly isolated microorganisms are *Pseudomonas aeruginosa (P. aeruginosa)* and *Staphylococcus aureus (S. aureus)*. Thus, biofilms are of importance not only due to their prevalence in HCAIs but also due to the increased antibiotic resistance that can be observed in comparison to their planktonic counterparts. Due to the importance of understanding biofilm interaction not only between bacterial species, but also interactions within the host, this research was focused on biofilm interactions using an *in vitro* co-culture with macrophage-like cells. Due to their prevalence in HCAIs, biofilms of *S. aureus, S. epidermidis,* and *P. aeruginosa* were explored. To combat antibiotic resistant microorganisms, research into alternative therapeutic treatments is of importance. One such alternative is cationic antimicrobial peptides. Currently, hCAP-18/LL-37 is the only known human cathelicidin with a broad spectrum anti-microbial response. Its antimicrobial and immunomodulatory properties make it of particular interest in antibiotic resistance research. This dissertation focuses on the development of a method to rapidly detect and quantify LL-37 in an *in vitro* co-culture setting.

Acknowledgments

I would like to thank the people who played an important role in the completion of this long journey. I first would like to thank my advisor Dr. Julie Stenken, who ensured the development of myself in research and critical thinking. Furthermore, Dr. Stenken made me see that grad school was an option, without her, I would not be here. I would also want to thank my committee members Dr. Jeannine Durdik, Dr. Ingrid Fritsch, and Dr. Jiangchao Zhao. Their support and advice over the years has been invaluable. Additionally, I would like to thank Dr. Jiangchao Zhao and Robert Story for the use of their laboratory for all bacteria and cell culture experiments. Furthermore, I would like to thank Dr. Josh Sakon for his willingness to help, and valuable advice, as well as Cody Brazel, M.Sc. for his training in gel electrophoresis techniques.

I am thankful for the past members of Dr. Stenken's lab that I had the privilege of working with. I am thankful for Dr. Alda Diaz Perez for her training in cell and biological work, and Dr. Taylor Snider Burnett for her support and encouragement in teaching aspirations. I also would like to thank the current grad students of the lab: Patrick Pysz, M.Sc. and Kenny Olubanjo, as well as the undergraduate students Sage Bentlage and Gillian Waters for being great colleagues and always available for a chat.

This journey has been long and hard, and would not have been possible without the support of friends and family. I am deeply grateful to Patrick Pysz for not only his hard work and willingness to 3D print various objects, but also his humor and availability for evening refreshments after a hard day. Without Dr. Alda Diaz Perez, I would not have made it this far. Not only for her mentorship in much of my research, but also always being down for smoothies and retail therapy. I know that I can always call both of these people for guidance and laughter.

Additionally, I would like to thank my family; my sister Brittany Lopez, for always being a phone call away during a mental breakdown, and constant stream of support and love, as well as my brother-in-law, Dr. Alex Lopez, for his guidance on this path, and being a sounding board for research ideas. Finally, I would like to thank my mother, Kimberly Gilson, who despite being gone, instills in me a drive that has allowed me to get this far.

Dedication

This dissertation is dedicated to my husband, Kyle Hunter. His unconditional love and support during these years has kept me going, despite multiple failures. Without him, I would not be here, and would have given up on my dreams of teaching.

Table of Contents

List of Tables

List of Figures

1 Introduction

1.1 Introduction

1.1.1 Healthcare-Associated Infections (HCAIs)

Healthcare-associated infections (HCAIs) are those that arise due to a patient receiving treatment in a healthcare setting. This includes acute-care hospitals, ambulatory care, long-term care, and family health clinics [1]. An HCAI occurs either 48 hours or more after admission, or within 30 days of healthcare treatment. According to the Centers for Disease Control (US), approximately 1.7 million hospitalized patients acquire a healthcare-associated infection each year, resulting in over 98,000 deaths and anywhere from $28.4 to $45 billion in treatment costs [2]. Furthermore, it has been established that more than 80% of these infections are caused by biofilms [3, 4]. While HCAIs can be bacterial, viral, fungal, or parasitic, there are between 12 and 17 species cause 80 to 87% of all HCAIs [1] . Among the most commonly isolated microorganisms are *Pseudomonas aeruginosa (P. aeruginosa)* and *Staphylococcus aureus (S. aureus)* [5, 6]. Biofilms are a collection of microorganisms surrounded by an extra-cellular matrix, as seen in Figure 1.1 below. They can be easily distinguished from their free-floating planktonic counterparts through the observable excretion of an extracellular substance, reduced growth rate, and alterations in gene expression [7]. Furthermore, biofilms can be comprised of more than one microbial species (poly-microbial), including interactions between multiple bacterial strains, and interactions between bacterial and viral strains, allowing for more diverse infection states [8].

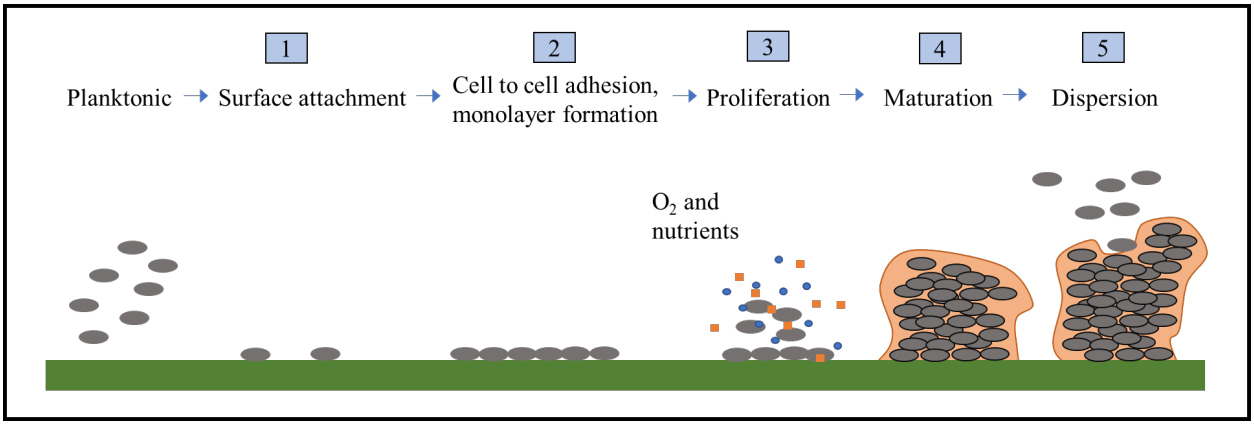


Figure 1.1 Biofilm Formation.

As Figure 1.1 shows, the formation of biofilms can be described in a 5-step process. The first part is free-floating planktonic bacteria adhering to a surface[9]. During the first stage of biofilm formation, adherence is reversible; this can also be known as primary adhesion. Many factors play a role in adherence such as nutrient availability, hydrodynamic forces, and the net sum of attractive and repulsive forces including electrostatic interactions, steric hinderance, and van der Waals forces [9-13]. This is followed by the cell-to-cell adhesion, allowing for the formation of a single layer of bacteria on the surface. At this stage, attachment is irreversible; this is due to the bacteria being able to withstand the shear forces in the microenvironment. Next, proliferation of the biofilm occurs. Oxygen and nutrients are diffused through the biofilm, allowing for growth. Fourth, a colony, or multilayer, forms and secretes an extracellular matrix, sometimes referred to as the glycocalyx; a biofilm is considered mature at this point [14]. The extracellular matrix is comprised of polysaccharides as well as carbohydrate-binding proteins, adhesive fibers, pili, and flagella for movement and increase adherence. Additionally, extracellular DNA provides a scaffold for the three-dimensional structure associated with mature biofilms. Overall, this extracellular matrix locks in nutrients and accounts for over 90% of all biomass. After biofilm

maturation, quorum-sensing molecules (QS) can leave and enter the biofilm, forming a chemical communication pathway between bacteria and other cells. Finally, dispersion occurs, where the biofilm expels more planktonic bacteria into the system, promoting further cycle initiation [7, 15-29]. Thus, biofilms are of importance not only due to their prevalence in HCAIs but also due to the increased antibiotic resistance that can be observed in comparison to their planktonic counterparts [14]. This inherent resistance causes numerous persistent and chronic infections [30]. Due to the importance of understanding biofilm interaction not only between bacterial species, but also interactions within the host, this research was focused on biofilm interactions using an *in vitro* co-culture with macrophage-like cells. Due to their prevalence in HCAIs, biofilms of *S. aureus, S. epidermidis,* and *P. aeruginosa* were explored.

1.1.2 Pseudomonas aeruginosa (P. aeruginosa)

Pseudomonas aeruginosa is a Gram-negative, rod-shaped, mono-flagellate bacterial strain commonly found in the healthcare setting. *P. aeruginosa* readily makes biofilms, which is in part due to the use of multiple attachment organelles to adhere to surfaces[31]. This includes both flagella and types IV pili-mediated twitching motility, allowing bacteria to attach more readily, move through the liquid interface and across the surface, as well as maintain adherence before irreversible attachment. Furthermore, *P. aeruginosa* is known to use cupA, part of the fimbrial gene clusters (cups), where fimbriae are involved in surface adherence and auto aggregation [32-37].

1.1.3 Staphylococcus aureus (S. aureus)

Staphylococcus aureus is a Gram-positive, spherical bacterial strain, also commonly found in HCAIs. *S. aureus* has shown antibiotic resistance since the 1940s, only two years after the discovery of penicillin. Additionally, by 1950, the prevalence of resistance became high enough,

that another drug therapy was required. By 1961, methicillin-resistant *Staphylococcus aureus* was found. *S. aureus* has many virulence factors including *Staphylococcal* siderophores, and pore-forming toxin alpha-hemolysin, allowing it to overcome the host's attempt at isolating metal ions, and access hemoproteins through the lysing of red blood cells, respectively [38-44].

The prevention and management of biofilm development in wounds is a very known priority in clinics and hospitals all around the world. Because of this, the study of biofilms is very important in the scientific community, in particular, biofilms from *S. aureus* and *P. aeruginosa*. *S. aureus* and *P. aeruginosa* are commonly isolated in cystic fibrosis (CF) and chronically infected wounds [45]. One strategy to study biofilm development is through their quorum sensing mechanism. After biofilm proliferation, the bacteria have the ability to communicate with each other through a quorum sensing mechanism (QSM). Quorum sensing is known to regulate the metabolic activity of the planktonic stage, induce microbial biofilm formation, and increase virulence [46].

1.1.4 Quorum Sensing (QS)

The quorum-sensing mechanism (QSM) is a chemical communication pathway involving molecules specific to various microorganisms; it is the process in which bacterial cells detect, produce, and respond through small diffusible signaling molecules that accumulate in the microenvironment [15, 47, 48]. The QSM allows for the synchronization of behavior in microorganisms depending on the population density, leading to biofilm formation, antibiotic resistance, and virulence [7, 15-18, 49]. This research is focused on both Gram-negative (*P. aeruginosa*) and Gram-positive (*S. aureus* and *S. epidermidis*) bacterial strains, which utilize differing quorum sensing molecules (QS) and pathways. Despite these differences, all QSMs incorporate three foundations: 1. Signaling molecules, known as autoinducers (AIs), are produced; 2. AIs are detected by receptor molecules in the cytoplasm or cell membranes; and 3. Upon

detection, AI production is activated, establishing a feed-forward loop [50]. However, *S. aureus* and *P. aeruginosa* are known to secrete and respond to different QS molecules. For example, Gram-negative bacteria like *Pseudomonas aeruginosa* use a quorum sensing mechanism involving four systems: las, rhl, pqs, and iqs, and can be described in Figure 1.2 [46].

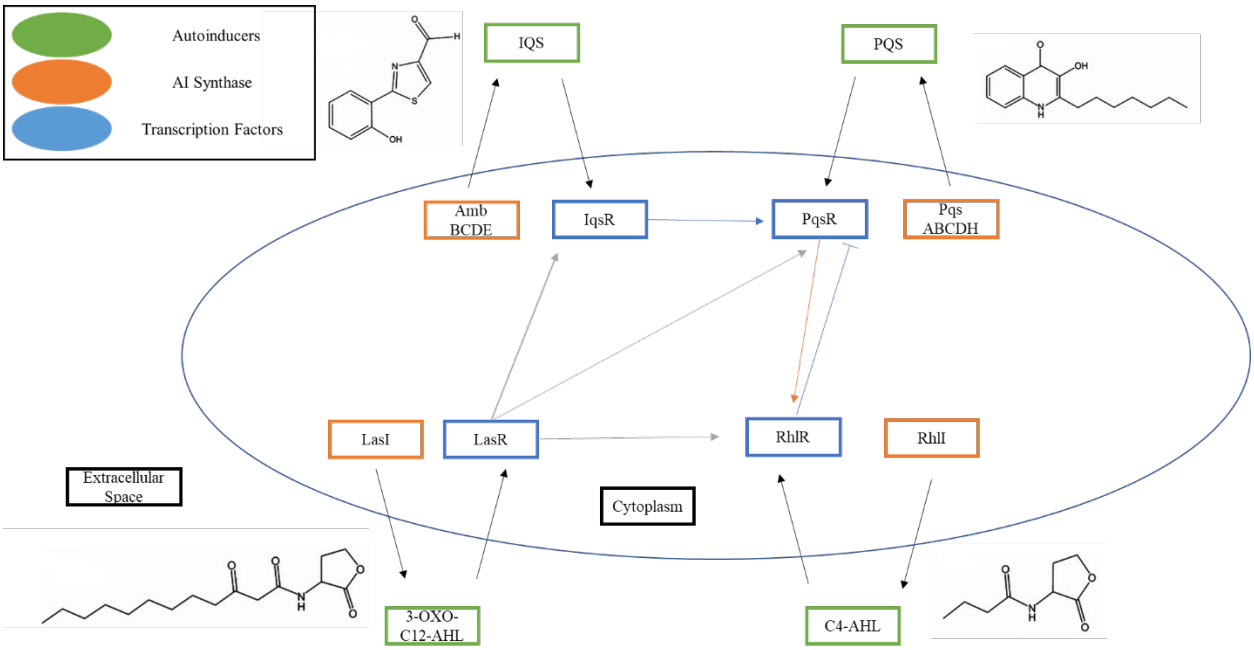


Figure 1.2 Quorum sensing for P. aeruginosa.

- The las system is controlled by the autoinducer N-(3-oxododecanoyl)-L-homoserine lactone (3-OXO-C12-AHL). LasR, the transcription factor in the las system homodimerizes after binding the autoinducer allowing for the connection of conserved promoters in target genes, which in turn promotes gene transcription [51].

- In a similar pathway, the rhl system is controlled by the autoinducer N-butryl-L-homoserine lactone (C4-AHL). This autoinducer binds the RhlR transcription factor in a similar pathway as the las system [51, 52].
- The pqs system is controlled by the autoinducer 2-heptyl-3-hydroxyl-4-quinolone, an alkylquinolone, also known as the *Pseudomonas* quinolone signal (PQS), as well as its precursor 2-heptyl-4(1H)-quinlolone (HHQ).[53] However, it should be noted, that while both HHQ and PWS can bind pqsR, HHQ binds with less robustness, approximately 100-fold [54]. Interestingly, PQS can function using both pqsR-dependent and pqs-independent pathways, through direct binding with previously unnoted protein partners in the cell, leading to several virulence pathways [51, 53-55]. Additionally, PQS has been found to bind RhlR, also of the QS mechanism. Through pqsH, HHQ can be converted into PQS; however, this transcription is controlled by LasR, interconnecting the two systems. This is of particular interest, as HHQ and PQS are specific to *P. aeruginosa*, but LasR applies to many forms of bacteria including *S. aureus*.
- The fourth system involved in the QSM is the iqs system, which is controlled by the autoinducer 2-(2-hydroxylphenyl)-thiazole-4-carbaldehyde (IQS). This synthesis comes from the non-ribosomal peptide synthase gene cluster ambBCDE. Disruption of transcription leads to a decrease in PQS and C4-AHL. Additionally, if LasI or LasR are disrupted, production of IQS stops, as well as the expression of ambBCDE [52]. Because of its ability to interfere with all three other systems, las is traditionally considered to be at the top of the QSM hierarchy [52]. These interconnected systems comprising the QS mechanism encode for various genes and virulence factors, a list of which, including effects on a host, can be seen in Table 1.1[56-63].

Table 1-1 Quorum sensing regulated genes, virulence factors, and their effects on hosts during an infection state [47-59].

QS System	Gene	Protein/Virulence Factor	Effect on Host System
Las	lasA	protease	Epithelial barrier disruption
	lasB	elastase	Degradation of matrix properties including elastin and collagen
	aprA	alkaline protease	Degradation of complement cascade and cytokines
	toxA	Endotoxin A	Cellular death
Rhl	lasB	elastase	Degradation of matrix properties including elastin and collagen
	rhlAB	rhamnolipids	Macrophage and lymphocyte (polymorphonuclear) necrosis
	phzABCDEFG	pyocyanin	Oxidative stress; delay in inflammatory response
	hcnABC	hydrogen cyanide	Arrest of cellular respiration
	lecA	lectin	Cytotoxicity; airway cilia paralysis
Pqs	phzABCDEFG, phzM,phzS	pyocyanin	Oxidative stress; delay in inflammatory response
	lecA	lectin	Cytotoxicity; airway cilia paralysis
	lasB	elastase	Degradation of matrix properties including elastin and collagen
	mexGHI-OpmD	efflux pump protein	Phenazine transportation
	rhlAB	rhamnolipids	Macrophage and lymphocyte (polymorphonuclear) necrosis
	lasA	protease	Epithelial barrier disruption
	hcnABC	hydrogen cyanide	Arrest of cellular respiration
	rsmAZ	Ribosomal RNA small subunit methyltransferase A	Coordinates "switch" between acute and chronic infection states; production of C4-AHL and 3-OXO-C12-AHL
	chiC	chitinase	Degradation of chitin

1.1.5 The immune system and macrophage response

The immune system is comprised of a collection of cells who function to protect the host from foreign bodies including debris from injury, as well as microbes that induce a viral, fungal, or bacterial infection. The immune response can be broken down into two subsets: innate and adaptive immunity. Monocytes and macrophages play a large role in both the innate and adaptive immune responses. Not only do macrophages phagocytize pathogenic material, but they also secrete cytokines and chemokines, which promote antigen presentation to T cells, triggering the adaptive immune response [64-66].

Monocytes arrive after neutrophils and eosinophils as part of the innate immune response and are converted to macrophages (MΦ) at the source of infection or injury [67, 68]. Monocytes derive from a progenitor called Macrophage Dendritic Cell precursor (MDP), which promotes differentiation into macrophages or dendritic cells (DCs) [69, 70]. Macrophages and DCs belong to a network called the mononuclear phagocyte system (MPS) and are crucial to both the immune and inflammatory responses [71, 72]. MΦ's are part of the innate immune system and are known as effector cells that sense the presence of invading pathogens and other danger signals in the microenvironment. Macrophages are heterogenous cells that can differentiate in various polarization states: M1, M2a, M2b, M2c, and M2d, allowing for altered responses to various stimuli; this allows for both responses to invasion and homeostasis [73].

1.1.6 M1 macrophages

M1 macrophages are known as classically-activated macrophages and are associated with a pro-inflammatory state. This stage of polarization is considered the "first line of defense" against intracellular pathogens and is related to the first segment of the wound healing cascade, also known as acute inflammation [74]. M1 macrophages are stimulated by bacterial components such as

lipopolysaccharide (LPS) and peptidoglycan, as well as cellular components of lysed cells, such as interferon-gamma (IFN-γ), and are primarily involved with phagocytotic activity, removing dead tissue from the wound area. Additionally, M1 macrophages play an important role in activating other immune cells by the release of cluster of differentiation 86 (CD86), reactive oxygen species (ROS), interleukin -1 (IL-1), IL-6, tumor necrosis factor-alpha (TNF-α), and IFN-γ [67]. M1 macrophages can be characterized through high levels of CD86 and varying levels of CD64 and CD80, dependent on stimuli. Furthermore, classically-activated macrophages promote the Th1 polarization of $CD4^+$ cells through the production of proinflammatory cytokines and chemokines, in particular, IL-12, INF-γ, and C-X3-C Motif Chemokine Ligand 1 (CX3CL1) [75]. Chronic or non-healing wounds remain indefinitely in the first stage of wound healing and can be characterized by low levels of T-cells and high levels of B-cells, however, as healing is promoted, macrophages polarize to an M2 phenotype [67].

1.1.7 M2 macrophages

M2 macrophages, also known as alternatively-activated macrophages are associated with anti-inflammatory characteristics, and the subsets of the M2 phenotype can be described through varying functionality and cytokine secretion levels. However, all M2 phenotypes are induced through Th2 responses and secrete high levels of IL-10, and low to null levels of pro-inflammatory cytokines such as IL-12 [76, 77].

M2a macrophages are induced through the presence of IL-4 and IL-13 and are characterized by high levels of CD86 and CD200R and low levels of CD14 and toll-like receptor 4 (TLR4). Additionally, they stimulate the production of IL-10, CCL13, CCL17, and CCL22 [74, 77, 78]. M2b macrophages are induced through LPS and IL-1β and are characterized by high levels of CD14 and CD80. This phenotype also produces proinflammatory cytokines, IL-10, and CCL1.

Lower levels of IL-12 and Human-leukocyte Antigen (HLA-DR), which constitutes a ligand for the T-cell receptor and acts as a marker for T-cell activation, are also indicative of M2b macrophages [75, 79-81]. Alternatively, M2c macrophages are induced by IL-10, glucocorticoids (GCs), or TGF-β, and can be characterized by high levels of CD163 and low levels of CD86 and HLA-DR [82, 83]. Additionally, they produce CCL18 and CCL16, and are associated with preventing tissue inflammation [74, 77, 78, 84]. M2d macrophages are also known as tumor-associated macrophages (TAMs) and are known to inhibit M1 macrophages. M2d macrophages are induced by IL-6 and monocyte-colony-stimulating factor (M-CSF) and CCL2 and contribute to tumor metastasis and angiogenesis, thus contributing to the inflammatory component of tumor tissues [74, 78]. M2d macrophages can be characterized by high levels of IL-10, and low levels of IL-12, TGF-β, C-X-C Motif Chemokine Ligand 10 (CXCL10), CXCL16, and C-C Motif Chemokine Ligand 5 (CCL5). A summary of the varying phenotypes and identifying characteristics can be seen in Figure 1.3 below. Through these mechanisms, macrophages are known to play an important role in the removal of pathogens and the elicitation of the inflammatory response during a pathogenic encounter. After the recession of pathogenic material, macrophages shift toward tissue repair and resolution of inflammation [82].

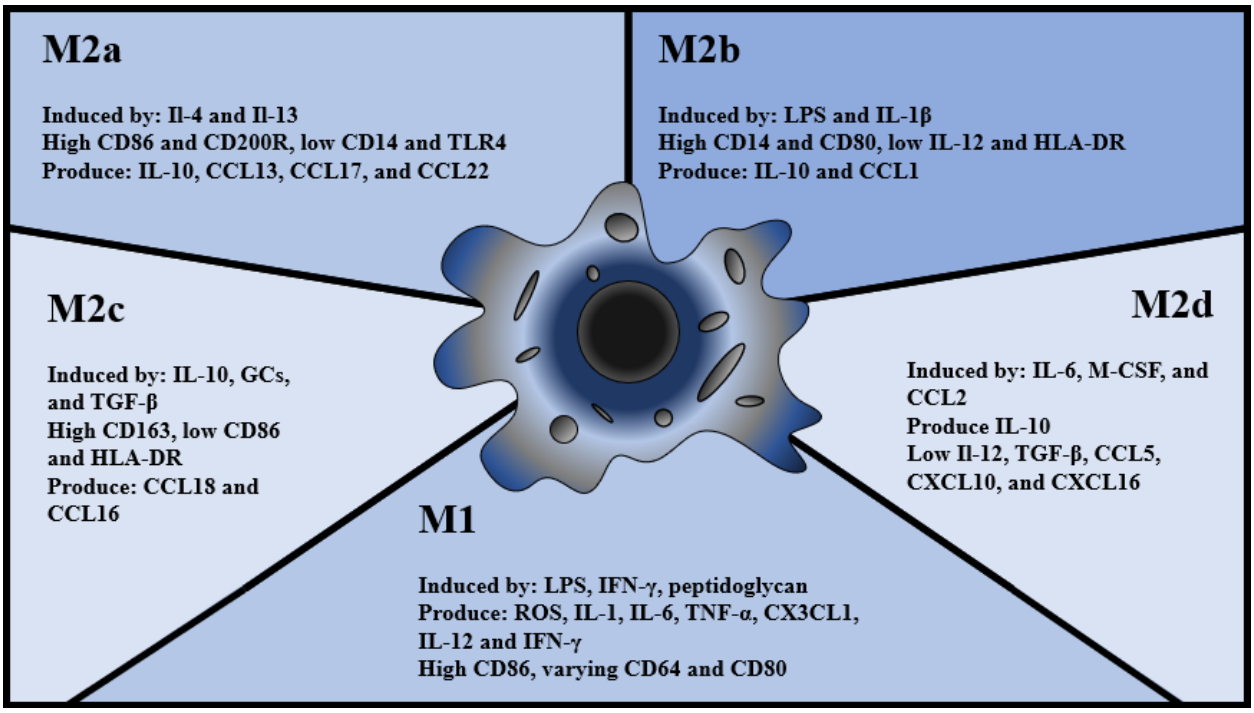


Figure 1.3 Macrophage Phenotypes

1.1.8 THP-1 cells

For this research, an *in vitro* setup will be used involving the culture of THP-1 cells. This is an immortalized monocytic cell line derived from the peripheral blood of an acute monocytic leukemia patient. Numerous studies have been performed on this cell line to determine its efficacy as a research model *in vitro* [85-89]. THP-1 cells can be differentiated into macrophage-like cells through the use of phorbol-12-myristate-13-acetate (PMA). The use of a PMA treatment activates protein kinase C (PKC), which increases the degree of differentiation of THP-1 cells. This is measured by the degree of adherence and surface marker expression [90-95].

1.1.9 Cathelicidins

Cathelicidins are host defense peptides that are known effector molecules of the innate immune system[96-101]. While amino acid sequence may vary, the commonality is maintained

through characteristics such as a positive charge state and amphipathicity, which allows peptides to interact with cell membranes which are usually anionic in nature, through the hydrophobic and hydrophilic nature of lipids [102-104]. Also known as cationic antimicrobial peptides (CAMPs), cathelicidins are known to play an important role in combating infection through the elimination of pathogens, in particular, Gram-negative and Gram-positive bacteria, such as *Pseudomonas aeruginosa* and *Staphylococcus aureus*, respectively. Cathelicidins are also known to have immunomodulatory functions such as directing chemotaxis, controlling both pro-inflammatory and anti-inflammatory states, and affecting cellular differentiation [4, 105]. The number of genes encoding for cathelicidins varies greatly, however in humans, there is only one. This gene is comprised of 4 exons: exon 1 encodes a 29-30 amino acid (AA) peptide for signaling. Exon's 2 and 3 encode for the conserved cathelin domain comprised of 99 to 114 amino acids. Finally, Exon 4 encodes the mature peptide of 12 to 100 AAs. This mature peptide possesses both the antimicrobial and immunomodulatory functions outlined above. Cathelicidins are stored inside granules until activation; this involves secretion, followed by the cleavage of the N-terminal pro-domain containing the cathelin domain, forming a biologically active peptide [103, 106-113].

1.1.10 Cathelicidin LL-37

LL-37 is a member of the CAMP family and is the only naturally occurring cathelicidin in humans. Currently, hCAP-18/LL-37 is the only known human cathelicidin with a broad spectrum anti-microbial response[114, 115]. Due to this, and its antimicrobial and immunomodulatory functions, it is of great research interest to many. This peptide is derived from the 18 kDa human cathelicidin hCAP18, wherein the C-terminal contains the 37-AA residues that are the biologically active form of the peptide known as LL-37 [116]. Moreover, LL-37 is known to be a chemoattractant immune effector cell that is known to modulate the production of a wide variety

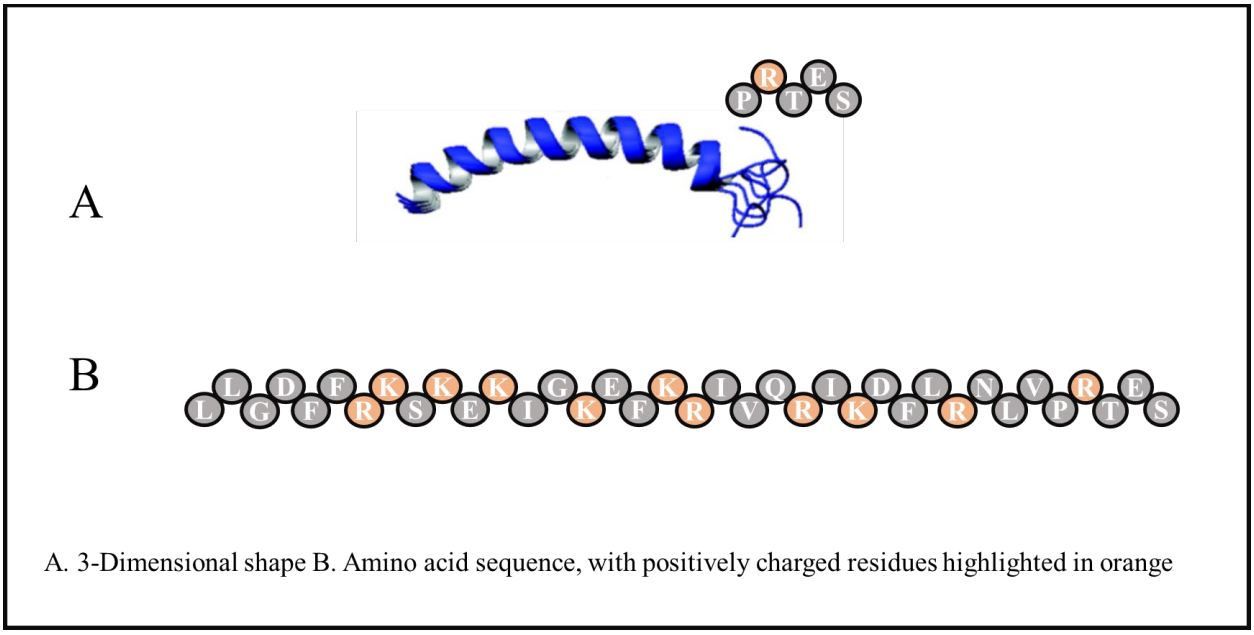
of inflammatory mediators [117]. LL-37 was shown to inhibit HIV-1 replication, through the down-regulation of HIV-1 chemokine receptors in an *in vitro* study [118]. Additionally, LL-37 has been shown to inhibit the LPS-induced pyroptosis of macrophages [119] Studies have also shown that LL-37 can act as a ligand for the integrin Mac-1 ($\alpha_M\beta_2$). Mac-1 interaction on macrophages, with LL-37 bound bacteria then promotes phagocytosis [120]. Furthermore, LL-37 is expressed in many places throughout the body, including the gastrointestinal and respiratory tracts, and leukocytes such as neutrophils, T-cells, natural killer (NK) cells, B cells, and monocytes. For a full list of LL-37 expressions throughout the human body, please see Table 1.2 below [121-123].

Table 1-2 Natural expression of LL-37 in the human body [124-147].

Cells	**Fluids**	**Tissues**
Leukocytes	Breast milk	Bone marrow
Myelocytes	Sweat	Skin of newborns
Metamyelocytes	Wound/Blister fluid	Nails (Keratin)
Spermatazoa	Nasal mucosa	Synovial membranes
	Bronchoalveolar lavage fluid	Developing Lung
	Saliva	Salivary glands
	Colon mucosa	Gingiva
	Seminal plasma	Squamous epithelia
	Vernix caseosa	Ocular surface epithelia
	Amniotic fluid	Lung Epithelia
		Colon epithelium
		Testis
		Epididymis epithelium

LL-37 is known to play roles in both adaptive and innate immunity through the regulation of monocyte differentiation into dendritic cells, induction of migration of monocytes, neutrophils, and T-cells, as well as the use of chemoattracts to modulate the production of various inflammatory factors. The structure of LL-37 is of importance when considering biological

activity and can be seen in Figure 1.4 below [148, 149]. LL-37 is an amphipathic α-helical peptide, comprised of a positive charge and hydrophobic residues at physiological pH. This allows for both the binding of the anionic cell membrane and the negative charge of the bacterial wall. Through this, LL-37 can disrupt the stability of the lipid bilayer, causing bacterial cell death [150, 151]. Conversely, studies have shown that pathogens such as Gram-negative bacteria can induce the expression of LL-37, through their LPS production. This can lead to further bacterial persistence and inflammation. To counteract this measure, LL-37 can bind LPS, preventing interaction with lipopolysaccharide-binding protein (LBP) and its co-receptor CD14. This in turn can neutralize the effect of the LPS [152-154].



A. 3-Dimensional shape B. Amino acid sequence, with positively charged residues highlighted in orange

Figure 1.4 LL-37 Structure. (A) is reprinted from *Biochimica et Biophysica Acta (BBA) - Biomembranes* "High-quality 3D structures shine light on antibacterial, anti-biofilm and antiviral activities of human cathelicidin LL-37 and its fragments,", vol. 1838, no. 9, pp. 2160-2172, 2014/09/01/ 2014, from G. Wang, B. Mishra, R. F. Epand, and R. M. Epand, with permission from Elsevier [155, 156]

While there are approximately 117 native fragments and synthetic analogs of LL-37 that have been studied for varying immunomodulatory and antimicrobial activities, LL-37 remains the most prevalently used cathelicidin for humans [157]. Varying activities can be caused by the degree of helicity. Fragments or analogs with higher degrees of helicity tend to have higher levels of antimicrobial activity [158]. For example, one analog of LL-37 has a helicity of 44.49 in comparison to LL-37's helicity of 5.08 and exhibits a high potency of antimicrobial activity. However, other fragments with lower helicities, 0.96 and 0.84, have a higher antimicrobial activity than LL-37 in regard to *E. coli* and methicillin resistant *Staphylococcus aureus* (MRSA) [158, 159].

Additionally, fragmenting LL-37 can result in a shift in function from signaling to antimicrobial activity. Research has shown that this can occur naturally *in vivo*. One study involving sweat found that degradation of LL-37 was occurring through the use of a serine-dependent mechanism. This mimics the way the hCAP 18 precursors are cleaved into its biologically active peptide [159-162].

1.1.11 Clinical significance

Cationic antimicrobial peptide LL-37 has been shown to be of clinical importance through multiple pathways. Due to its overall positive charge and amphipathic alpha helical structure, LL-37 can disrupt the integrity of the lipid bilayer of the negatively charged cell wall and anionic cell membrane, through opsonization. Studies have shown that LL-37 augments both Gram-negative and Gram-positive bacterial phagocytosis through binding of integrin Mac-1 on macrophages. However, direct bactericidal activity of this peptide varies depending on sampling medium; in low salt conditions, strong bactericidal activity is seen, however, in physiologically relevant mediums, a much lower level of direct phagocytosis is seen, indicating that this is not

the primary function of LL-37[120]. Further research has shown that LL-37 induces the migration of monocytes and macrophages to sites of inflammation and injury, as well as neutrophil activation through Mac-1 dependent binding. Additionally, it has been shown that LL-37 promotes macrophage differentiation to a pro-inflammatory state through this process[163]. Thus, LL-37 can modulate the gene expression of cytokine and chemokine receptors, which can in turn interact with bacterial quorum sensing molecules, such as N-3-oxo-dodecanoyl-L-homoserine lactone[164, 165]. Due to the interaction of all these mechanisms, a method to detect and quantify LL-37 in an in vitro co-culture of macrophage-like cells and bacterial biofilms, is needed to further elucidate infection progression and potential therapeutic treatments.

1.2 Methods for Separation and Quantification

Numerous methods have been used for the detection and quantification of LL-37 including enzyme-linked immunosorbent assay (ELISA), western blot, and polymerase chain reaction (PCR), as well as liquid chromatography-mass spectrometry, which can be used all of which come with varying advantages and disadvantages [166, 167].

1.2.1 Enzyme-linked immunosorbent assay (ELISA)

Enzyme-linked immunosorbent assay is a highly specific and accurate method for the detection and quantification of a multitude of antibodies, antigens, proteins, glycoproteins, and hormones, and is commonly considered the gold standard of immunoassays. All of the available ELISAs for LL-37 are sandwich-based assays, involving both an antibody coating (primary or capture antibody) and a detection antigen (secondary antibody). One of the advantages of the ELISA is the sensitivity of these assays ranges from 0.039-0.94 ng/mL, using 100 uL sample volumes. Studies have shown that ELISA can be used in the detection and quantification of LL-37 including research in ovarian cancer, lupus erythematosus, rheumatoid arthritis, ear wax, and

colon epithelial cells [168-171]. However, the disadvantage of ELISAs is the time of the run because each assay run takes approximately 6-7 hours to run. Additionally, each kit is rather costly in comparison to other methods for quantification. However, given the number of samples that can be analyzed at a time, and the lack of a need to purify biological samples before processing, is also of great advantage to an ELISA.

1.2.2 Gel Electrophoresis (SDS-PAGE)

Sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) is a common separation technique used on complex mixtures of proteins. It involves denaturing proteins prior to a gel run, which can make it not desirable depending upon the application. Through the use of a molecular weight ladder, gel electrophoresis can be used to detect and identify unknowns in a sample. In order to quantify levels of these proteins, gels can be cut and trypsinized, wherein the cells retain characteristic molecular markers and undifferentiated cellular morphology. Trypsin is considered the gold standard in protein digestion of peptides. Thereafter, samples can be quantified using HPLC-MS [172, 173]. Studies have shown the use of SDS-PAGE in the detection of LL-37 in human plasma, cystic fibrosis sputum, and bronchoalveolar lavage (BAL) fluid [174-176]. Even though SDS-PAGE can be used to easily visualize the presence of LL-37, SDS-PAGE is a very time-consuming method.

1.2.3 Western Blot

Western blot is a common technique used for the separation and identification of proteins. This process involves an agarose gel electrophoresis, followed by a transfer onto a membrane, and finishing with incubation with antibodies specific to the analyte of interest [177]. Western blot is highly specific through the use of antibodies, however, on its own, it is not quantitative. Several studies have shown western blot to be an effective method in the detection of LL-37 in whole

saliva, mitochondria, and type I insulin-like growth factor receptor (IGF-1R) [178-180]. In order to quantify analytes, HPLC-MS is commonly used. Due to the nature of this technique, it is very time-consuming and is not quantifiable as a stand-alone method. For this reason, the western blot is a very costly and time-consuming experiment.

1.3 Dissertation Aims

The study of LL-37 is of research importance due to its ability to manipulate broad-spectrum anti-microbial properties and promote immune activation due to the induction of chemokine production. Studies have shown that LL-37 directs macrophage differentiation to a pro-inflammatory phenotype through the induction of chemokine production and the leukocytes' recruitment [117, 181]. It is involved with the regulation of monocyte differentiation into dendritic cells, induction of migration of monocytes, neutrophils, and T cells. Furthermore, LL-37 uses chemoattracts to modulate the production of various inflammatory factors and can disrupt both the anionic cell membrane and the negatively charged bacterial cell wall, thus causing bacterial cell death [150, 151]. As it is the only naturally occurring cathelicidin in humans and is expressed in many places throughout the body, including the gastrointestinal and respiratory tracts, and leukocytes such as neutrophils, T cells, natural killer (NK) cells, B cells, and monocytes, a method to rapidly and accurately detect and quantify LL-37 is needed [121, 122]. Studying LL-37 can elucidate further treatment options for HCAIs and antibiotic-resistant infections.

The research objectives were to:

- Develop a sample preparation method for the detection of LL-37 using a spiked standard to allow a reproducible measurement for future analytical validation methods.
- Develop an analytical method using an HPLC-MS/MS protocol for the detection and quantification of LL-37 in a spiked standard sample for future *in vitro* analysis.

- Analyze LL-37 levels using the newly developed method in an *in vitro* setting: the *in vitro* method consisted of THP-1 activated co-culture with bacterial biofilms of *S. aureus, S. epidermidis,* and *P. aeruginosa*.

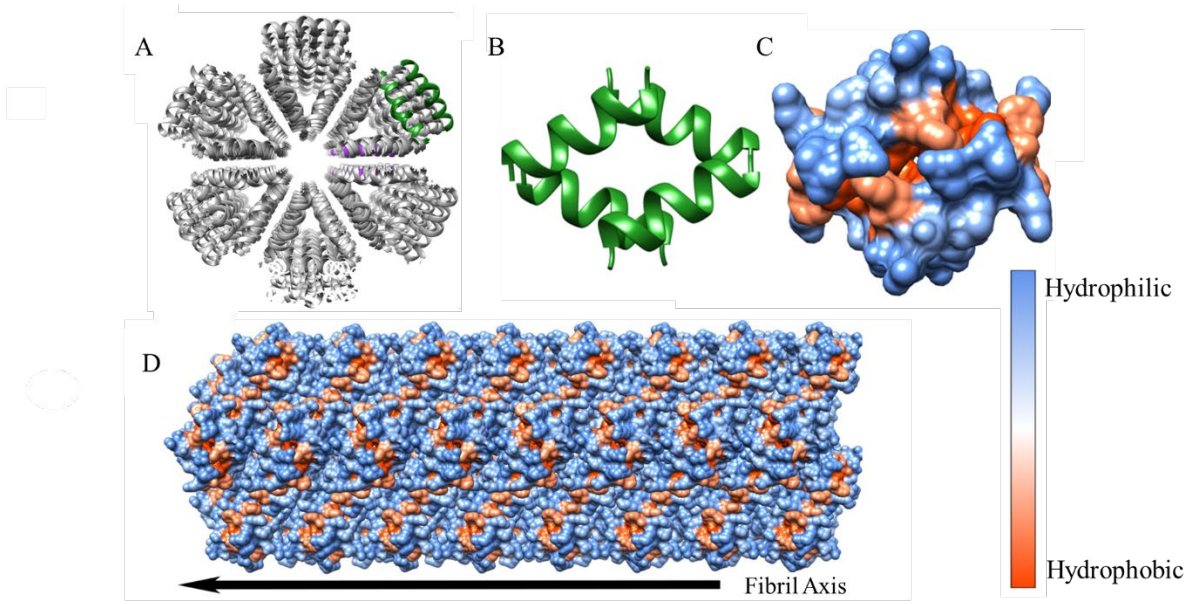
2 Development of a sample preparation technique for the extraction of LL-37 from spiked-standards

2.1 Introduction

LL-37 is a cationic antimicrobial peptide of 37 amino acids, proteolytically cleaved from the precursor hCAP18. As it is the only naturally occurring cathelicidin in humans and has both antimicrobial and immunomodulatory effects, it is of particular interest to the research community. Different studies have shown the natural expression of LL-37 in leukocytes, in particular monocytes and macrophages [121, 122]. As LL-37 can play roles in both adaptive and innate immunity, through the regulation of monocyte differentiation, and chemoattractant use to modulate the production of various inflammatory factors, a method to detect and quantify LL-37 is of particular interest for potential therapeutic use [117]. Due to the importance of LL-37, a method for the detection and quantification of LL-37 has shown to be important for the study of the protein in the immune system during an infection and the possibility of a therapeutic method. However, the chemical properties and structure of LL-37 provides a drastic challenge to method development.

LL-37 is an α-helical peptide comprised of a positive charge and hydrophobic residues at physiological pH. This allows for both the binding of the anionic cell membrane and the negative charge of the bacterial cell wall [150, 151]. Through this, LL-37 can disrupt the stability of the lipid bilayer, causing bacterial cell death. However, LL-37 is also known to aggregate in solution. At lower concentrations (0.5 μM to 1.5 μM), it is found as a mix of monomers and dimers, however, at higher concentrations (50 μM) it can form trimers [182, 183]. Moreover, this self-assembly into different polymeric states can affect how LL-37 interacts with pathogens and the immune system, altering its antimicrobial and immunomodulatory effects [156, 182-185].

Variations in structure can lead to reproducibility and stability issues. Further research into LL-37 interactions has found that this peptide is comprised of a hexameric fibril with a central pore that can be seen below (Figure 2.1). This chapter explores peptide stability in various solvents, and in a time-dependent manner, to establish consistent, reproducible measurements in LL-37 spiked standards.



A, The assembly is shown as grey ribbons, with two representative four-helix bundles colored green and purple to emphasize orientation in the fibril. **B**, An isolated four-helix bundle shown as green ribbons. **C**, The four-helix bundle, in the same orientation as in **B**, shown in a surface representation colored by hydrophobicity, according to the scale bar. **D**, The surface representation rotated by 90º, colored by hydrophobicity.

Figure 2.1 Supramolecular Structure of LL-37 [185]. Reprinted from *Nature Communications,* vol. 11, no. 1, p. 3894, 2020/08/04, from Engelberg and M. Landau, "The Human LL-37(17-29) antimicrobial peptide reveals a functional supramolecular structure."

2.2 Materials and Methods

2.2.1 Materials and Reagents

High-performance liquid chromatography (HPLC)-grade water, dimethyl sulfoxide (DMSO), methanol (MeOH), formic acid (FA), and acetonitrile (ACN) were obtained from Fisher Scientific (Hanover Park, IL). LL-37, L-Arginine monohydrochloride, and urea were obtained from Sigma-Aldrich (St. Louis, MO). Guanidine hydrochloride was obtained from MP Biomedicals (Solon, OH). HEPES, free acid was obtained from BDH (West Chester, PA). N,N,N',N'-Tetramethylethylenediamine (TEMED) was obtained from TCI (Portland, OR). Tris-base, ammonium persulfate (APS), glycerol, and bromophenol blue were obtained from VWR (Solon, OH). Acrylamide was obtained from Alfa Aesar (Ward Hill, MA). Acetic acid was obtained from Supelco (Burlington, MA). Coomassie Brillant Blue R-250 was obtained from AMRESCO (Solon, OH). Glass spacer plates (0.75mm) and short plates were obtained from Bio-Rad (Hercules, CA).

2.2.2 Sample preparation for solid phase extraction (SPE) analysis:

2.2.2.1 Development of a method for sample preparation

To determine if solid-phase extraction was a viable sample preparation method, several techniques were evaluated such as solubility, evaporation, and sample reconstitution. The two main spiked standards to evaluate sample preparation were: (1) 0.1% v/v formic acid (FA) in acetonitrile (ACN) and (2) 0.1% v/v FA in HPLC grade water. Several other solvents were used to study the solubility and evaporation study.

2.2.2.2 Solvent Effect on Solubility and evaporation analysis

To study the solubility of LL-37 using a wide variety of solutions, standards of 10 ppm LL-37 were made in the following solvents:

I. 0.1% v/v formic acid (FA) in acetonitrile (ACN).

II. 2% v/v DMSO in 60% v/v HPLC grade water with 0.1% v/v FA in ACN.

III. 3% v/v DMSO in 40% v/v HPLC grade water with 0.1% FA in ACN.

IV. 4% v/v DMSO in 60% v/v HPLC grade water with 0.1% FA in ACN.

V. 6% v/v DMSO in 40% v/v HPLC grade water with 0.1% FA in ACN.

After the sample was prepared using each different solvent, each sample was measured using HPLC/MS using different time points of 0, 18, and 24 hours. Another important technique for the sample preparation to run SPE is the evaporation technique. For this technique, standards of 10 ppm LL-37 were made using 0.1% v/v FA in ACN, placed in HPLC vials, at room temperature, and weighed over 24 hours.

2.2.2.3 Sample Reconstitution

To study the stability of LL-37 during reconstitution, a spiked standard was prepared using 10 ppm LL-37 standards in either 0.1% v/v formic acid (FA) in HPLC-grade water, or 0.1% v/v FA in acetonitrile (ACN). Another spiked standard was prepared and passed through the SPE cartridge. The elution of the cartridge and the standard samples were dried using a CentriVap Vacuum at room temperature until dry. Samples were then reconstituted in 0.1% v/v FA in HPLC grade water or 0.1% v/v FA in ACN and measured using HPLC/MS.

2.2.3 Sample preparation for gel electrophoresis

Another method that was studied simultaneously from SPE cartridge sample preparation was gel electrophoresis; gel is a common sample preparation for the detection and quantification of a wide variety of proteins, using HPLC-MS. The gel was used for the extraction of LL-37 from a complex matrix and then HPLC-MS was run for the detection and quantification of the compound.

2.2.3.1 Gel electrophoresis analysis (PAGE)

For this set of experiments, spiked standards of 1 mg/mL LL-37 were dried under a nitrogen stream and reconstituted in either L-Arginine (0.5M and 1M), guanidine hydrochloride (0.1M, 0.25M, and 0.5M), or urea (0.1M and 0.5M) in water. Two buffers were prepared for the gel analysis: the loading buffer and the running buffer: the running buffer for the gel run and the loading buffer for the sample run. The running buffer for the gel was prepared using 1.5% w/v Tris-base and 4.2 % w/v HEPES free acid to be utilized in the gel and loading buffer formulations. The loading buffer was made by diluting the running buffer in water (1:5), and combing 10% v/v running buffer, 30% v/v glycerol, 2% v/v 0.5% bromophenol blue in water. After the spiked standard and the buffers were prepared, the gel was prepared. For this, a 15% w/v crosslinked native polyacrylamide gel was made by combining 24.8% v/v running buffer, 49.6% v/v acrylamide, 0.058% v/v N,N,N',N'-Tetramethylethylenediamine (TEMED), and 0.695% v/v 10% ammonium persulfacte (APS), in water, and pipetting into 0.75mm glass sandwiches. To run samples, the standard spiked samples were mixed in a 1:2 ratio with loading buffer, and vortexed for 20 seconds. Then, the spiked samples were loaded into the Mini-PROTEAN 3 Cell gel system, which was run at 200 V for various times, including 35 minutes (plus 10 minutes at 100 V), 45 minutes, 5 hours, and 8 hours, with voltages, flipped on a Bio-Rad PowerPac Basic. After the run, the gel was introduced in a fixing solution for 3 mins. The fixing solution was made with 40% v/v methanol and 10% v/v acetic acid in water. After the 3 mins in the fixing solution, the gel was transferred into a staining solution for 1 min which contained 0.25% w/v Coomassie Brillant Blue R-250, 45% v/v methanol, and 10% acetic acid in water. Later after the staining solution, the gel was transferred to a destain solution overnight. The destain solution was prepared with 5% v/v

methanol and 7.5% v/v acetic acid in water. Gels were then analyzed using ImageJ software to determine distance traveled, in order to investigate possible deaggregation compounds.

2.2.4 Mass spectrometry analysis (MS)

For the MS analysis, two methods were performed for the detection and quantification of LL-37. These two methods were evaluated to obtain high sensitivity and selectivity of the analysis. The selected method is described in Chapter 3 of this dissertation. For Chapter 2, UPLC-MS/MS analysis was used to determine the fragmentation of LL-37. For experimental analysis, the Bruker-Daltonics HPLC/MS was used.

2.2.4.1 UPLC-MS/MS analysis

UPLC-MS/MS analysis was performed using a Shimadzu UPLC-20A/HPLC-30A in line with a Shimadzu 8060 triple quadrupole mass spectrometer with a heated electrospray source (positive-ion mode). UPLC-MS/MS was performed using a C18 column (2.1 × 150 mm, 1.7 μm particle size, ACQUITY UPLC Peptide BEH C18 with pore size 300 Å)) with a linear gradient composed of 0.1% formic acid (FA) in HPLC-grade water with 0.1% FA in acetonitrile ramped at a rate of 7.5 (v/v%) acetonitrile/min over 10 min. The flow rate was 0.3 mL/min. Sample volumes of 1 μL were injected. The three most intense multiple reaction monitoring (MRM) fragments from protonated intact precursor ions for intact LL-37 were first fully optimized at their corresponding retention time windows using Shimadzu Lab solution software, Version 5.89. From the three MRM channels, the MRM event corresponding to the most intense fragment ion was denoted the "quant" ion (749.8 m/z), and the other two fragment ions (642.7 and 899.5) were used as reference ions for identification purposes. Reference ion ratios were calculated compared to the "quant" ion, and unknown samples were required to meet within 30% of the reference ion ratio of the standard and ± 0.2 min of the retention time of the standard. This is the default compound identification

criteria that Shimadzu lab solution software adopted from European Union standards [186-189]. LL-37 standards were analyzed three times [149].

2.2.4.2 HPLC-MS

HPLC-MS analysis was performed using a Hewlett Packard (Palo Alto, CA) 1100 series HPLC with a Bruker-Daltonics Esquire (Billerica, MA) 2000 quadrupole ion trap mass spectrometer with an electrospray source (positive-ion mode). Separations were performed using a C8 column (4.6 × 150 mm, 5 μm particle size, Supelco, Discovery BIO Wide Pore) with a linear gradient composed of 0.1% v/v formic acid (FA) in HPLC-grade water with 0.1% v/v FA in acetonitrile ramped at a rate of 5.67 (v/v%) acetonitrile/min over 15 min. The flow rate was 0.7 mL/min. Sample volumes of 10 μL were injected.

2.2.5 Data Analysis

All LL-37 spiked samples and measurements were performed in triplicate and reported as mean ± SD. A student's t-test was performed on samples in triplicate or higher to determine statistical significance. Image J software was used on scanned gels to determine the distance traveled.

2.3 Results and Discussion

2.3.1 Sample preparation analysis of LL-37 in mass spectrometry (MS)

For the sample preparation analysis for the detection and quantification of LL-37 in mass spectrometry, two methods were studied that are well-known for the extraction of protein for analysis. One of them is the use of gel electrophoresis and the other one is the use of an SPE cartridge. In our lab, SPE cartridges have been used for the extraction of lipids for HPLC-MS/MS analysis.

2.3.2 Gel Electrophoresis (PAGE)

Gel electrophoresis (PAGE) was studied for the extraction of LL-37 for the detection and quantification of complex samples. Native polyacrylamide gel electrophoresis (PAGE) is commonly used to determine peptide aggregation in solution and was used to determine aggregation of LL-37 [190, 191]. Due to the hydrophobic nature of the running solvent, LL-37 standards were dried under a nitrogen stream, before being reconstituted in Milli-Q water. Stand-alone LL-37 standards acted as a control and were diluted to 1:4 in loading buffer. LL-37 standards in L-arginine were diluted 1:2. Variations in dilution ratios comes from the resistance of stand-alone LL-37 to run down a gel. Polyacrylamide native gels were made with 15% w/v crosslinking and run at 200V. Due to the positive nature of the peptide, gels were run from positive to negative voltages, allowing the peptide to run down the gel using its natural charge. One challenge in the extraction of LL-37 is the formation of protein aggregates and misfolds that could reduce the extraction and absolute recovery. L-arginine has been used as an additive to prevent protein refolding which leads to aggregation suppression [192]. Previous studies have shown that 0 – 2 M of L-arginine has shown effective for a decrease of protein refolding, and an increase of stability and shelf-life [192-196]. For this part of the experiment, it was proposed that L-arginine could decrease the LL-37 aggregation, resulting in a more consistent sample over-time.

Lane	Sample
1-3	0.5 M L-Arginine
5	1 mg/mL LL-37 Standard
6	0.5 M L-Arginine in 1 mg/mL LL-37
7	1 M L-Arginine in 1 mg/mL LL-37
8	1 mg/ mL LL-37 Standard
9	0.5 M L-Arginine in 1 mg/mL LL-37
10	1 M L-Arginine in 1 mg/mL LL-37

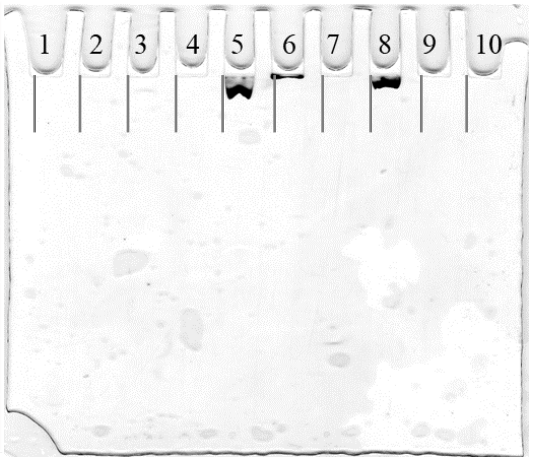


Figure 2.2 Native PAGE with 15% crosslinking. 200V for 45 min, with L-arginine and LL-37 standards.

Lanes 1-3 in Figure 2.1 are 0.5M L-arginine to ensure proper gel running, and lanes 5 and 8 are 1mg/mL LL-37 standard to act as a control. Two concentrations of L-arginine were used in conjunction with 1 mg/mL LL-37 standards: 0.5 M (lanes 6 and 9) and 1 M (lanes 7 and 10). From Figure 2.1, it was established that the L-arginine passed through the gel, while the samples of just LL-37 (lanes 5 and 8) barely moved down the gel in 45 minutes. While 45 minutes was the standard run time for smaller peptides such as LL-37, it was proposed that a longer run time was necessary to achieve band separation. For this reason, Figure 2.2 shows an increase of time to 5 and 8 hours of run time.

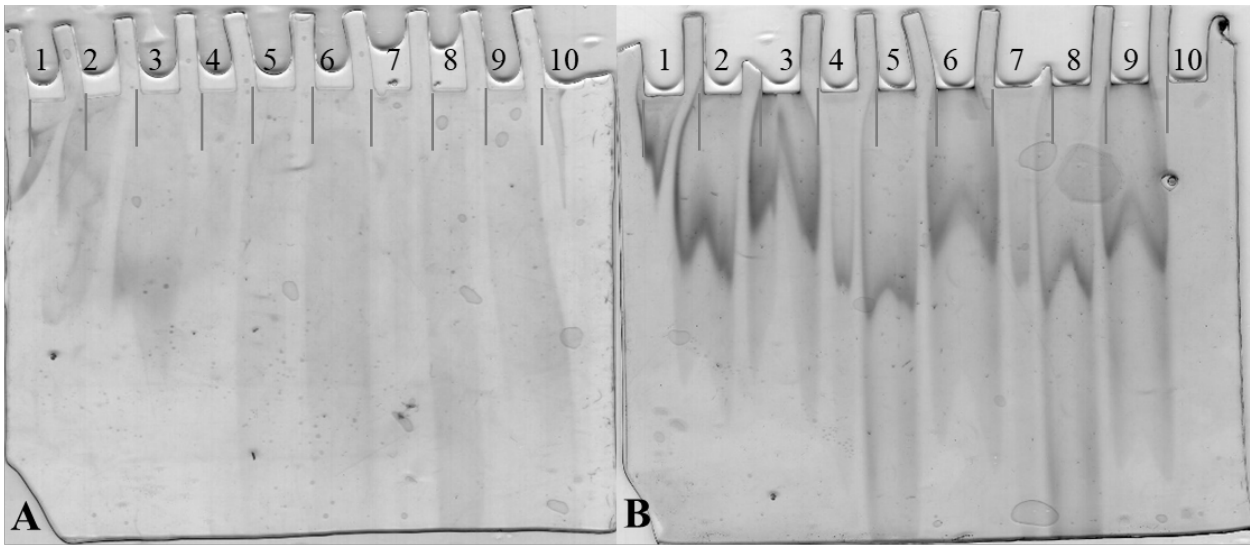


Figure 2.3 Native PAGE with 15% crosslinking, with L-arginine and LL-37 standards. A) 200 V for 8 hours, B) 200 V for 5 hours.

Figure 2.8 shows gel electrophoresis run over 8 (A) and 5 (B) hours. For both gels, the distribution of the lanes was the same for consistency:

- Lanes 1,4, and 7 are 1 mg/mL of LL-37 standard.
- Lanes 2, 5, and 8 are 0.5 M L-arginine in 1 mg/mL LL-37.
- Lanes 3, 6, and 9 are 1M L-arginine in 1 mg/mL of LL-37.

Figures 2.1and 2.2 A and B show that varying running time results in observable differences, such as a sample run through each lane. To compare Figure 2.2 (A) and (B), there is a more subtle difference. While increasing the gel run time helped the samples travel further down the gel, the 8-hour run time led to band broadening and spreading. While decreasing the run time to 5 hours helped in the resolution of band broadening effects, they were still too prevalent to determine peptide aggregation. It was determined that the time needed to be decreased further.

A

Lane	Sample	Distance (mm)
1	250 ppm LL-37 Std	7.254
2	0.5 M Arginine in 1 mg/mL LL-37	0.465
3	1 M Arginine in 1 mg/mL LL-37	71+
4	250 ppm LL-37 Std	4.1
5	0.5 M Arginine in 1 mg/mL LL-37	1.04
6	1 M Arginine in 1 mg/mL LL-37	71+
7	250 ppm LL-37 Std	4.8
8	0.5 M Arginine in 1 mg/mL LL-37	0.868
9	1 M Arginine in 1 mg/mL LL-37	71+

B

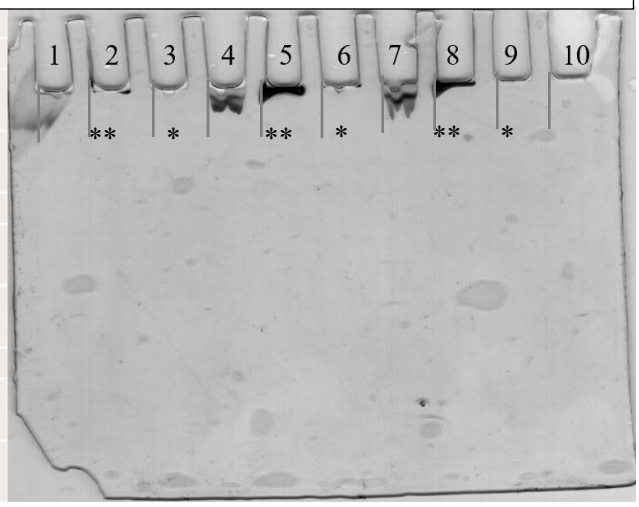


Figure 2.4 (A) Description of Native PAGE in (B), and (B) Native PAGE with 15% crosslinking, with L-arginine and LL-37 standards, 200 V 35 min, 100 V 10 min.

Figure 2.3 (B) shows that 1M L-arginine (lanes 3,6, and 9) is a viable candidate for decreasing peptide aggregation, which could lead to a consistent HPLC/MS run-time. This was confirmed with the calculation of the distance travel of the compound inside of the gel. Figure 2.9 (A) showed that the distance travel was 71+ mm for lanes 3, 6, and 9. This method involved a run time of 35 minutes at 200 V, followed by 10 minutes at 100 V, and was used in further gel electrophoresis experiments. After studying L-arginine, guanidinium chloride (GdnHCl) and urea were also explored to determine if sample denaturation could lead to an increase in consistency between samples over time for an HPLC/MS run. In the study, GdnHCl and urea were studied using a variety of concentrations and the distance travel was calculated for each experiment. The distance traveled was calculated to obtain the optimized sample preparation of the extraction of LL-37 using Native PAGE. After the extraction, the LL-37 could be detected and quantified using HPLC-MS or HPLC-MS/MS.

A

Lane	Sample	Distance (mm)
1	250ppm LL-37 Std	2.067
2	0.1 M Guanidinium chloride in 1mg/mL LL-37	16.781
3	0.25 M Guanidinium chloride in 1mg/mL LL-37	16.024
4	0.5 M Guanidinium chloride in 1mg/mL LL-37	15.842
5	250ppm LL-37 Std	9.920
6	0.1 M Guanidinium chloride in 1mg/mL LL-37	18.852
7	0.25 M Guanidinium chloride in 1mg/mL LL-37	18.938
8	0.5 M Guanidinium chloride in 1mg/mL LL-37	17.236

B

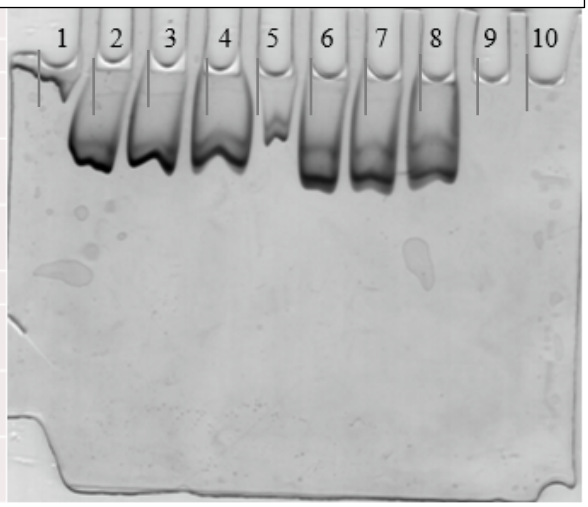


Figure 2.5 (A) Description of Native PAGE in (B) and (B) Native PAGE with 15% crosslinking. 200V for 35 min., 100V for 10 min, with Guanidinium chloride (GdnHCL) and LL-37 standards.

Guanidinium chloride (GdnHCL) and urea are commonly used denaturing agents for proteins. However, the mechanism through which they denature varies. To illustrate, GdnHCl produces a denaturing effect through favorable interaction with polar sections of proteins. Here, the non-polar side chains have little to no interaction with the denaturing agent. Studies have shown that urea acts as a denaturant through the formation of hydrogen bonds with peptide groups[197]. This was observed through the analysis of peptides and their ability to block acid- and base-catalyzed peptide hydrogen exchange. Due to variations in the mechanisms of denaturation, both GdnHCl and urea were chosen as candidates for peptide stability over time [198-208].

A B

Lane	Sample	Distance (mm)
1	250ppm LL-37 Std	0.706
2	250ppm LL-37 Std	1.058
3	0.1 M urea in 1mg/mL LL-37	5.645
4	0.5 M urea in 1mg/mL LL-37	5.381
5	0.1 M urea in 1mg/mL LL-37	6.439
6	0.5 M urea in 1mg/mL LL-37	7.233
7	0.1 M urea in 1mg/mL LL-37	7.850
8	0.5 M urea in 1mg/mL LL-37	8.203
9	250ppm LL-37 Std	0.970

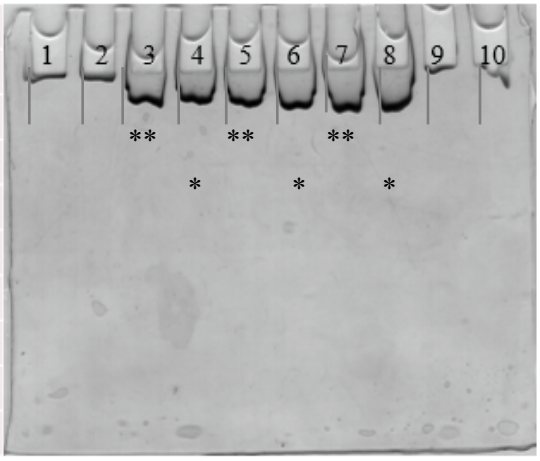
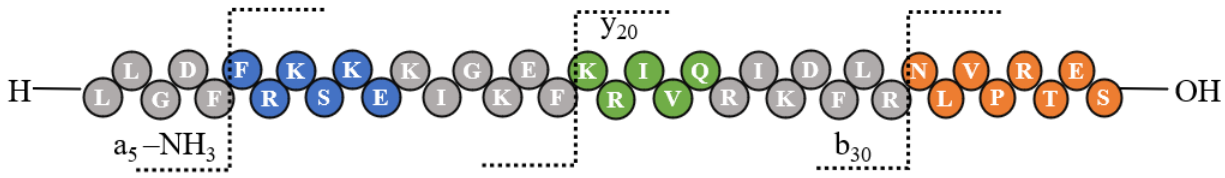


Figure 2.6 (A) Description of Native PAGE in (B) and (B) Native PAGE with 15% crosslinking. 200V for 35 min., 100V for 10 min, with urea and LL-37 standards.

In analyzing Figures 2.4 and 2.5, denaturing the samples of LL-37 with either guanidinium or urea increased sample travel down the gel, as compared to the standard LL-37. However, it is proposed that due to the highly polar nature of LL-37, the interaction mechanism of GdnHCl was more effective. Due to the promising results that with the addition of GdnHCL, spiked standards of 1mM GdnHCl in conjunction with both 10 ppm and 1ppm LL-37 were run using HPLC-MS. This experiment was important to observe if there was consistency between the samples at different time points. 0 and 24 hours; For solubility reasons, the guanidinium-LL-37 mixture was prepared using 5% FA in 60% ACN (v/v%). For LL-37 quantification, standards of LL-37 (10 ppm) were analyzed using UPLC-MS/MS, with the three most intense multiple reaction

monitoring (MRM) fragments from protonated intact precursor ions for intact LL-37, used as the "quant" and reference ions, as outlined in Table 2-1 below.

Table 2-1 Multiple reactions monitoring (MRM) channels used in LL-37 identification and quantification.



LL-37 Amino Acid Sequence	Quant Ion/Ratio	Ref Ion 1/Ratio	Ref Ion 2/ Ratio
Possible amino acid sequence	FRKSKE	KRIVQ	NLVPRTES
Mass to charge ratio	749.8 m/z	642.7 m/z	899.5 m/z
Ratio	100%	51%	18%
Tentative chemical formula was used for the formulation of this table. Percent of ratio was approximated for the quant ion and reference ions.			

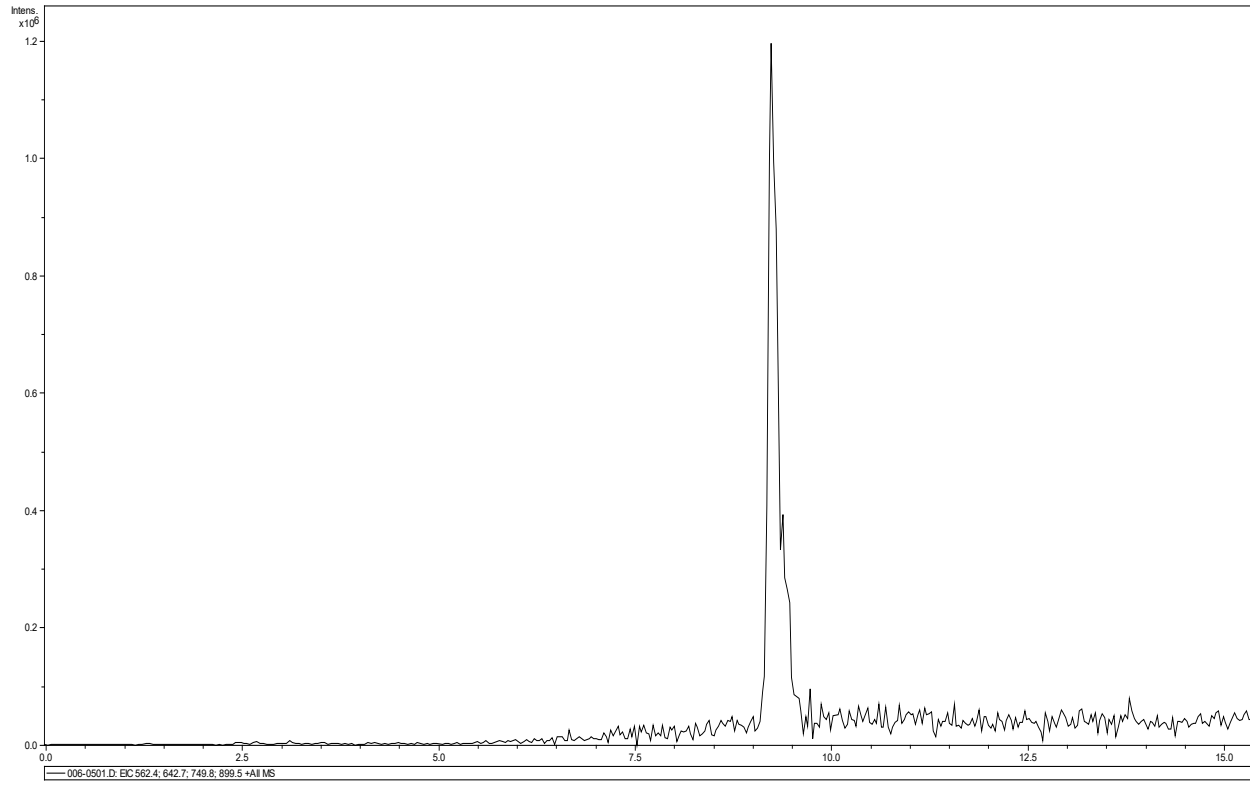


Figure 2.7 (A) EIC of LL-37 Standard (10 ppm).

Figure 2.7 outlines the extracted ion chromatogram (EIC) for the LL-37 standard. Peaks correlating to the mass-to-charge ratios of 562.4,642.7,749.8, and 899.5 m/z were analyzed with 749.8 m/z denoted as the “quant” ion, and 642.7 and 899.5 *m/z* as the reference ions, as can be seen in Figure 2.8.

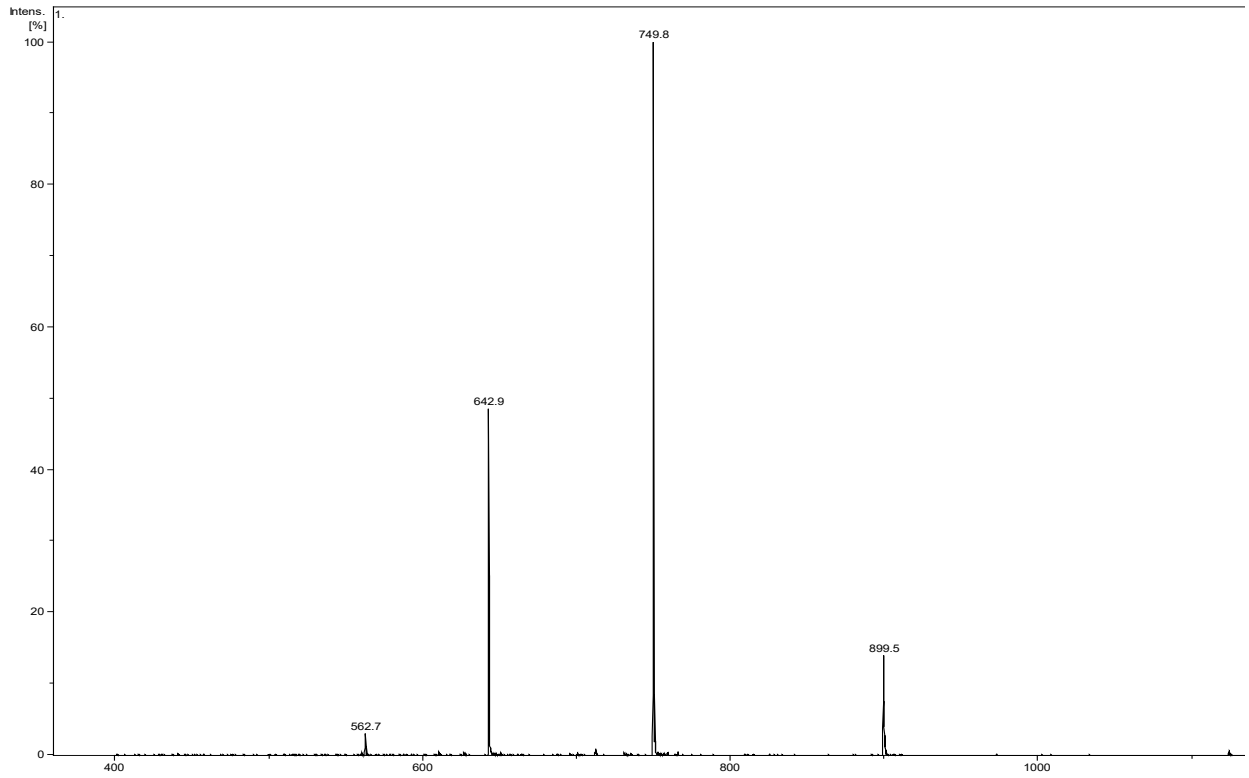


Figure 2.8 Mass spectrum for selected peaks with mass-to-charge ratios of 562.4, 642.7,749.8, and 899.5 m/z.

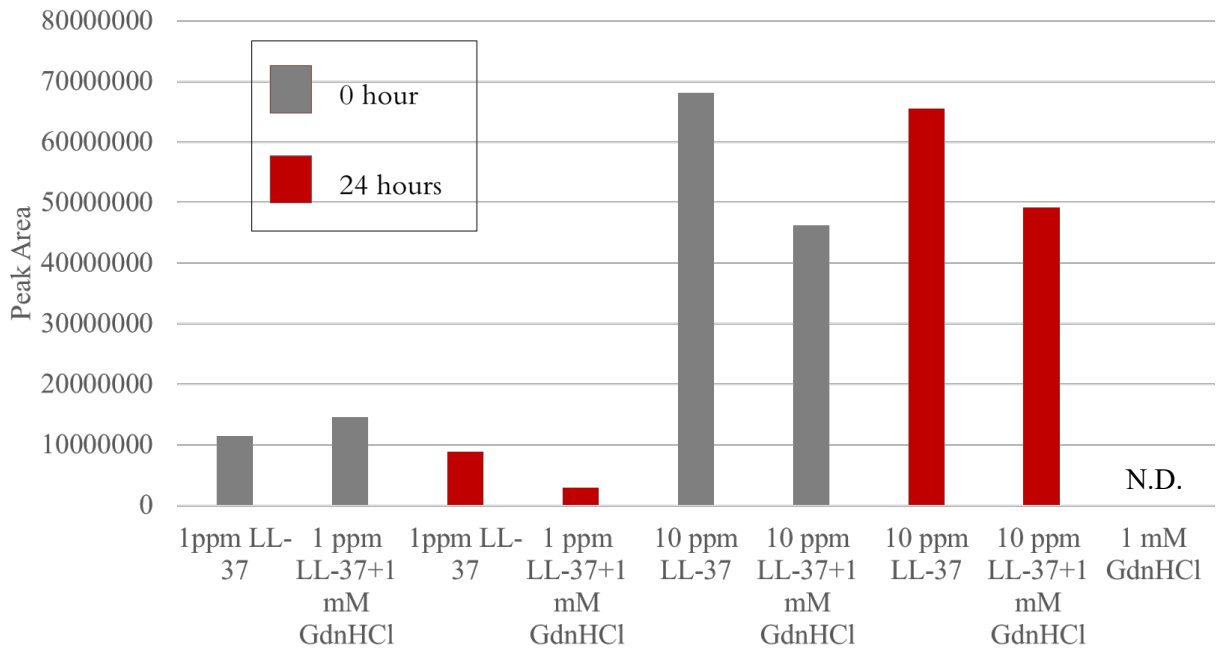


Figure 2.9 HPLC/MS data for LL-37 spiked standards with 1 mM guanidinium chloride at 0 and 24 hours at room temperature. Moreover, for solubility purposes of both GdnHCl and LL-37, a solvent of 5% formic acid (FA) in 60% (v/v) of acetonitrile (ACN) was used.

Figure 2.8 shows two concentrations of LL-37, 10 ppm, and 1 ppm, in conjunction with 1mM GdnHCl over the course of 24 hours. An observable decrease in peak area between standards and samples containing guanidine hydrochloride, as well as an inability of the addition of GdnHCl to provide a more consistent sample over time, resulted in it being discarded as a viable candidate in reproducibility. However, in analyzing the standards over time in Figure 2.8, it was determined that the new solvent of 5% v/v FA in 60% v/v ACN resulted in a more consistent sample over time. Rather than peak area increasing over time, as previously seen, which is indicative of a solubility issue, these samples decreased slightly over time, as can be expected with protein

samples. Because these samples were left at room temperature overnight, moving forward, samples were placed at -20 ºC, prior to analysis to reduce degradation rates.

2.3.3 Sample preparation for the extraction of LL-37 for SPE analysis

For the sample preparation of LL-37 using solid-phase extraction (SPE), there are a few steps that are very important to increase the absolute extraction efficiency. Sample reconstitution, solubility study, and evaporation analysis were studied as the first part in determining a sample preparation method for LL-37. Several of these initial sample preparations are presented in this chapter; however, the optimized sample preparation, detection method, and calculations are presented in Chapter 3 of this dissertation.

2.3.3.1 Sample drying and reconstitution

A method of using HPLC/MS to measure quorum sensing molecules has been previously established in the laboratory. Previously, SPE elutes were dried in a CentriVap vacuum chamber, then reconstituted in the same elution solvent, 0.1% v/v FA in ACN. However, inconsistencies in data led to the need to test standards in various solvents to explore the possibility of increasing solubility and reducing carryover effects. For this experiment, several ratios of dimethyl sulfoxide (DMSO) and acetonitrile were prepared:

- 2% v/v of DMSO in 60% v/v acetonitrile in HPLC grade water
- 3% v/v of DMSO in 40% v/v acetonitrile in HPLC grade water
- 4% v/v of DMSO in 60% v/v acetonitrile in HPLC grade water
- 6% v/v of DMSO in 40% v/v acetonitrile in HPLC grade water

Using the four preciously mentioned solutions, a spiked standard of 10 ppm of LL-37 was prepared and compared with the original solvent of 0.1% v/v FA in ACN, using HPLC/MS over

3-time points: 0hr, 18hr, and 24hr, the results of which are shown below (Figure 2.8). In this experiment, DMSO was chosen as a supplemental solvent due to its common use in the mobile phase during HPLC/MS to increase sample recovery. Moreover, DMSO is commonly used in storage of screening collections in the pharmaceutical industry, and has been previously used in cleaning the HPLC/MS between runs, where residual LL-37 is removed from the column [209-212].

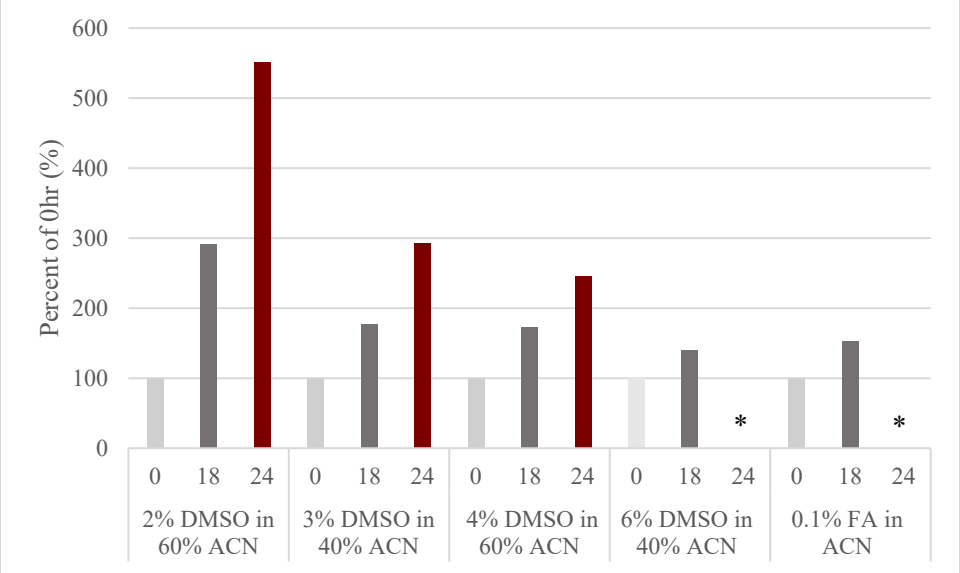


Figure 2.10 HPLC/MS data for 10 ppm LL-37 standards in 5 solvents that are described in the heading of the graph was run over time, denoted in relation to the percentage of the initial value (0 hr) * indicates power failure; n=1 for preliminary data.

From Figure 2.9, it is clear that repeat measurements of the same sample do not give rise to repeat results as expected. Rather, there is an increase of signal over time, with samples more than doubling over a 24-hour window. A possible challenge of using DMSO as an elution solvent is the evaporation; for this reason, an evaporation analysis was run.

2.3.3.1.1 Evaporation Test

Before selecting a solvent, it is important to study the evaporation of the solution. Knowing that volatile solvents can quickly evaporate at room temperature, it was necessary to determine if the results in Figure 2.9 was due to solvent evaporation over time. To do this, 3% DMSO in 40% 0.1% FA in CAN (v/v) was picked as a "middle-ground" between the above solvents, and 100 µL was sealed in an HPLC vial and massed over 24 hours to determine volume loss to the air. Furthermore, to establish if the experimental run time could be shortened to solve this issue, this experiment was focused on the first 6 hours of sample evaporation, as seen in Figure 2.9 below.

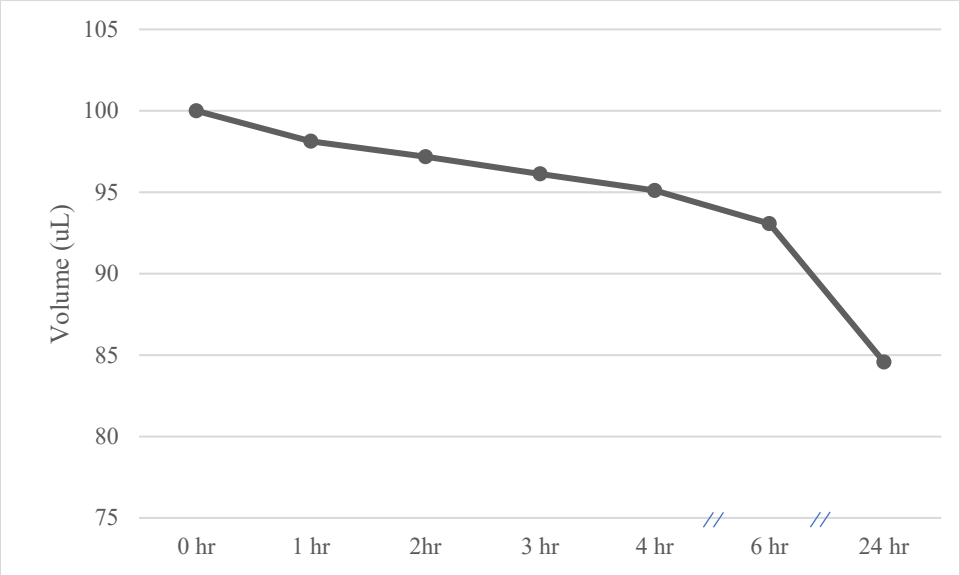


Figure 2.11 Solvent evaporation over time, using 3% DMSO in 40% ACN (v/v).

While some volume loss was observed, it does not account for the doubling of the intensity of LL-37 standards over 24-hours as seen in Figure 2.9. As it is known that LL-37 aggregates in solution into multimers, it was determined that the samples may be aggregating, and thus, gel electrophoresis was investigated to determine if peptide aggregation could be solved as Figure 2.4, Figure 2.5, and Figure 2.6 showed. DMSO could have been a possibility; however, drastic changes

in sample concentration led to the need to re-evaluate sample reconstitution steps. For this reason, several solvents were studied, and absolute recovery was calculated in Figure 2.11.

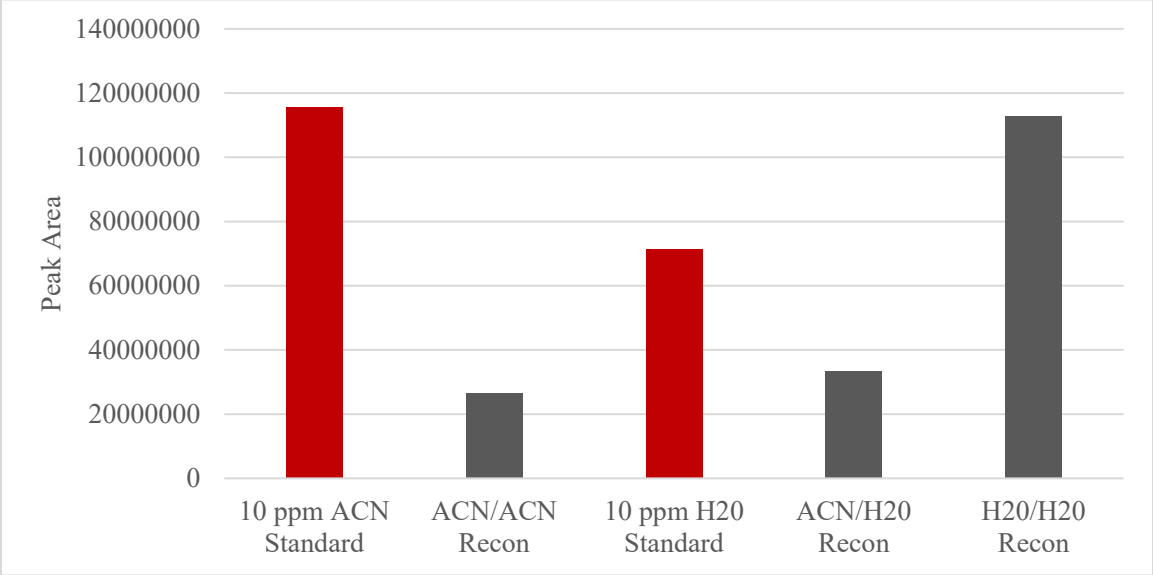


Figure 2.12 10 ppm LL-37 standards (red) in either 0.1% FA in HPLC grade water or 0.1% FA in ACN and their dried and reconstituted counterparts (grey) were measured using HPLC/MS.

Standards of 10 ppm (parts per million) of LL-37 in either 0.1% FA in HPLC grade water or 0.1% FA in ACN were analyzed through direct injection and reconstitution using HPLC/MS. Figure 2.11 shows differences between direct injection and reconstitution. ACN resulted in a recovery of 23%, and those that were reconstituted in HPLC grade water had a varying effect. The ACN standard reconstituted in water resulted in a recovery of 47%, whereas the HPLC-grade water standard reconstituted likewise gave a recovery of 158%. Given the greater than 100% recovery, the water standard was run again, 6 hours after the initial injection. As shown in the extracted ion chromatogram (EIC) correlating to 562.4 m/z, 642.7 m/z, 749.8 m/z, 899.5 m/z, and 1124.1 m/z for 10 ppm LL-37 in HPLC grade water, signal intensity is increasing over time. At a time point of 6 hours, the peak area is 253% as compared to t=0 hours (Figure 2.11).

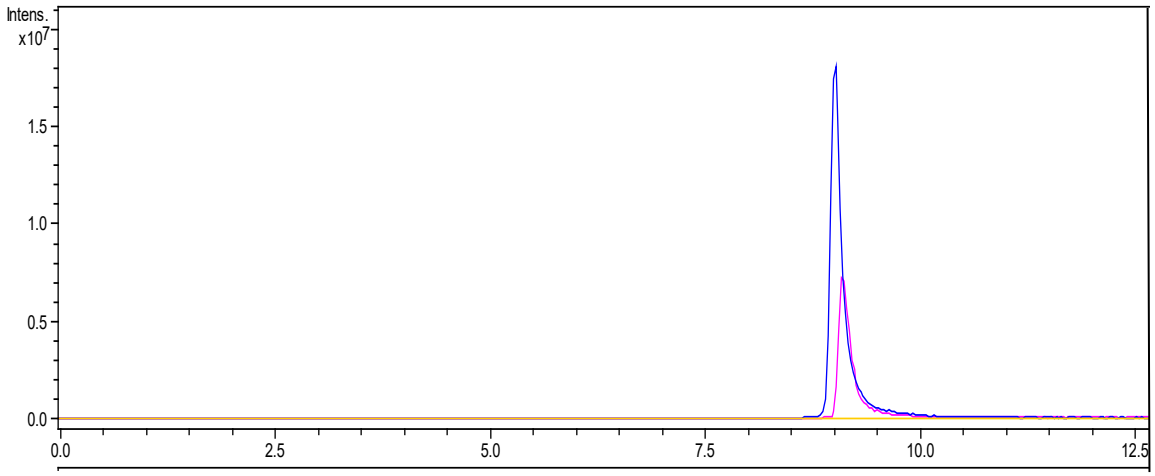


Figure 2.13 EIC of precursor ions for 10 ppm LL-37 in HPLC grade water at t=0 (pink), and t=6 hours (blue). The yellow line indicates DMSO injection.

Using the data in Figure 2.12, it was determined that a solubility issue was causing the increase overtime. Moving forward, LL-37 standards are of a much lower concentration (1ppm and lower), are used for sample analysis.

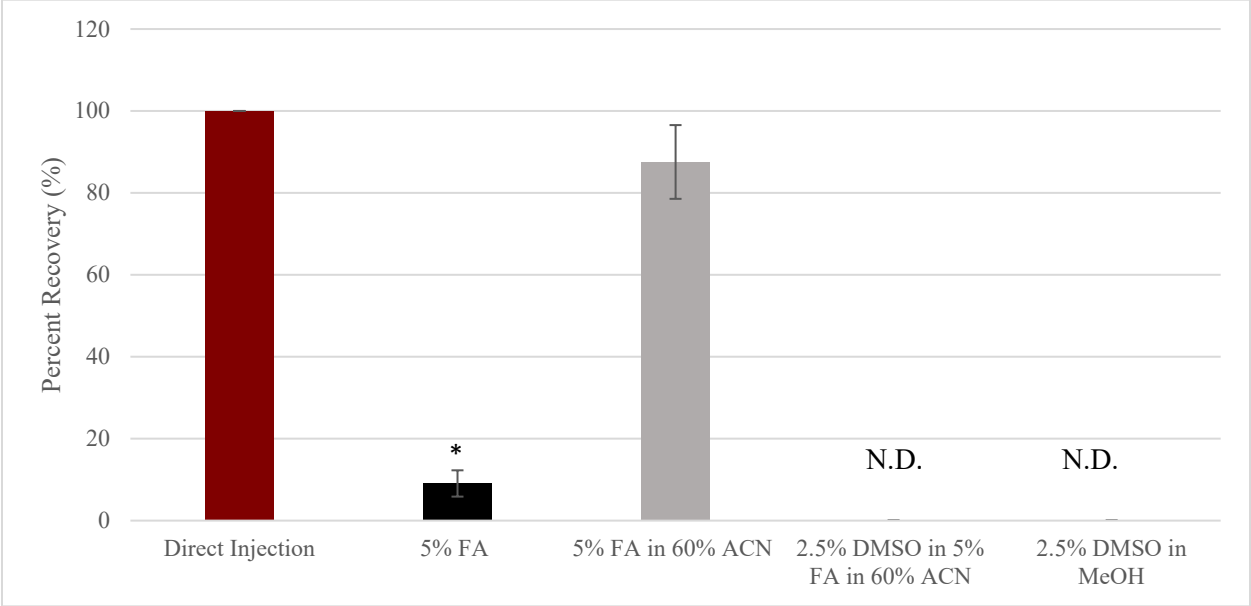


Figure 2.14 Percent recovery of reconstituted solid-phase extraction (SPE) samples were calculated using a 10 ppm of LL-37 and a wide-variety of solvents, including The solvents used were 5% (v/v) FA in HPLC grade water, 5% v/v of FA in 60% v/v of ACN, 2.5% DMSO in 5% FA in 60% ACN (v/v), and 2.5% v/v of DMSO in methanol.

Figure 2.14 explores alternative reconstitution solvents in relation to a direct injection of the sample. Solid-phase extraction (SPE) samples were dried in a Centrivap vacuum chamber and reconstituted in either 5% formic acid, or 5% FA in 60% acetonitrile. While the formic acid (black) alone resulted in an average percent recovery of 9.1±6.4%, as compared to the direct injection (red), the 5% FA in 60% ACN (v/v) (grey) resulted in an average percent recovery of 87.5±18.0%. In this experiment, it was possible to observe that 5% of FA in 60% of ACN (v/v) is a viable solution for the reconstitution phase.

2.4 Conclusion

The purpose of this work was to establish a method for consistently reproducible sample preparation over time. Previous work into LL-37 resulted in nonreproducible measurements, with HPLC/MS data giving rise to peak areas that did not correlate with sample concentrations. Due to the formation of multimers in solution, native gel electrophoresis was explored to determine if L-arginine, urea, or guanidine hydrochloride could provide viable options to de-aggregate the solution. While promising using gel electrophoresis, when analyzed on the HPLC/MS, GdnHCl proved not to be a viable candidate for maintain sample consistency over-time. Various solvents for standards of LL-37 such as water, ACN, and DMSO were explored to determine if solubility could be increased. It was established that a solvent of 5% FA in 60% ACN (v/v) was a viable option for maintaining sample consistency over time, as well as for sample reconstitution. Chapter 3 will use results obtained from Chapter 2 to explore a solid-phase extraction method for complex samples.

3 Development and validation of a solid-phase extraction method to extract LL-37 from spiked standards

3.1 Introduction

Solid-phase extraction is a commonly used method to purify and extract analytes of interest from biological samples, such as urine or plasma, prior to HPLC or GC/MS analysis [213-215]. This allows for removal of interfering biological matrix components, through washing of cartridges, while also allowing for analyte enhancement, as elution phases require small volumes, and can be further preconcentrated by evaporating organic solvents. SPE is a common sample preparation technique; some previous work focuses on the use of solid-phase extraction techniques for analyte preparation including cyanobacteria tainted wastewater, as well as for the extraction of allura red in beverages and food [216, 217]. This work was aimed at developing a solid-phase extraction method for the purification of complex biological samples in the form of a co-culture comprised of macrophage- like cells and bacterial biofilms. LL-37 spiked standards were used to develop a viable method before analysis of biological co-cultures in Chapter 4. Multiple solid-phase extraction cartridges were chosen in an attempt to extract LL-37. Bond-Elut PPL, a polymer-base cartridge, was chosen for its common use with both hydrophobic and hydrophilic analytes, as LL-37 is known to by hydrophilic. These have also been used previously in the laboratory, in extracting quinolones and lactones from a complex co-culture. SOLA WCX was also tested, as it is a mixed-mode ion-exchange cartridge, and LL-37 is known to have a positive charge as physiological pH (isoelectric point or pI of 10.61) [186-188, 218]. Finally, C8 SPE cartridges were tested as it was proposed that sample aggregation found in Chapter 2, was contributing to incomplete elution in the other cartridges.

3.2 Materials and Methods

3.2.1 Materials and reagents

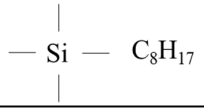
High-performance liquid chromatography (HPLC)-grade water, methanol, formic acid, ammonium hydroxide, and acetonitrile were obtained from Fisher Scientific (Hanover Park, IL). Bond-Elut-PPL with a 50 mg and 100 mg bed capacity as well as C8 SPE cartridges with a 50 mg bed capacity were obtained from Agilent Technologies (Santa Clara, CA). C18 spin column was obtained from G-biosciences (St. Louis, MO). SOLA WCX SPE cartridges (10 mg/1mL) were obtained from Thermo Fisher Scientific (Waltham, MA). Roswell Park Memorial Institute (RPMI-1640) medium and Hank's solution were obtained from American type culture collection (ATCC) (Manassas, VA). Fetal bovine serum (FBS), antibiotic and antimycotic solution (100x), and LL-37 were all obtained from Sigma-Aldrich (St. Louis, MO) LL-37 was also obtained from Bachem (Torrance, CA).

3.2.2 Solid-Phase extraction

LL-37 was extracted with a Bond-Elut PPL, SOLA WCX, or C8 SPE cartridge. Different procedures were performed using SPE to determine which method was most suited to isolate LL-37 from the spiked standards. The Bond-Elut PPL cartridge is comprised of a styrene-divinylbenzene cross-linked copolymer modified with a proprietary non-polar surface, which allows for the retention of highly polar analytes. SOLA WCX cartridges are a mixed-mode SPE cartridge, meaning that it is comprised of both a reverse-phase chemistry, in this case a proprietary formula, along with weak-cation exchange, also proprietary; however, its functional group is known to be carboxylic acid, which can be helpful at extracting highly polar analytes. C8 SPE cartridges were also tested, as the shorter hydrocarbon chain can be useful when a C18 cartridge

retains an analyte too strongly, not allowing for effective elution. A diagram of the various SPE cartridge sorbent structures can be seen below (Table 3.1) [219, 220].

Table 3-1 SPE phase and sorbent structure

SPE Cartridge	Base	Structure
Bond-Elut PPL	Polymer-based	$[CH_2CH(C_6H_5)]_x[CH_2CH[C_6H_4(CHCH_2)]]_y$ + proprietary non-polar surface
SOLA WCX	Polymer-based WCX	Proprietary structure + -COOH
C8	Silica-based	

Using a previously established procedure in the laboratory, Bond-elut PPL, SOLA WCX, or C8 SPE cartridges were washed with 2 mL methanol with 0.1 % v/v formic acid. Then, the column was pre-conditioned with 2 mL of HPLC-grade water with 0.1 % v/v formic acid, before 2 mL of sample was added to the SPE column. The column was washed with 2 mL of HPLC-grade water with 0.1% v/v formic and the sample was eluted with 2 mL of acetonitrile. Additionally, each cartridge was used to extract LL-37 from 10 ppm standards, using the same procedure as outlined above, however, the elution solvent was changed to one of the following: 5% FA in 60% ACN, 5% FA, 5% FA in ACN followed by 2.5% DMSO in 5% FA in 60% ACN, 5% FA in 60% ACN followed by 50% DMSO in methanol, 5% FA in ACN followed by 2.5% DMSO in methanol, or 5% FA in 60% ACN followed by 50% DMSO in 5%FA in 60% ACN. Finally, SOLA WCX SPE cartridges were used to extract LL-37 from standards using the manufacturer's method, as follows: Cartridges were washed with 500 µL of methanol and conditioned with 500 µL 1% NH_4OH in HPLC-grade water, before 500 µL of standard treated with 1% NH_4OH was added. The

cartridge was washed with 500 µL 1% NH_4OH in HPLC grade water, and then 500 µL of 500 µL 1% NH_4OH in methanol. Samples were eluted with 500 µL 1% FA in methanol.

3.3 Preliminary data

HPLC/MS analysis was performed using a Hewlett Packard (Palo Alto, CA) 1100 series HPLC with a Bruker Daltonics Esquire (Billerica, MA) 2000 quadrupole ion trap mass spectrometer with an electrospray source (positive-ion mode). Separations were performed using a C8 column (4.6 × 150 mm, 5 µm particle size, SUPELCO, Discovery BIO Wide Pore) with a linear gradient composed of 0.1% formic acid (FA) in HPLC-grade water with 0.1% FA in acetonitrile ramped at a rate of 5.67 (v/v%) acetonitrile/min over 15 min. The flow rate was 0.7 mL/min. Sample volumes of 10 µL were injected.

3.4 Data Verification

3.4.1 HPLC-MS/MS Analysis

HPLC-MS/MS analysis was performed using a Shimadzu UPLC-20A/HPLC-30A in line with a Shimadzu 8060 triple quadrupole mass spectrometer with a heated electrospray source (positive-ion mode). Separations were performed using a C8 column (4.6 × 150 mm, 5 µm particle size, Supelco, Discovery BIO Wide Pore) with a linear gradient composed of 0.1% formic acid (FA) in HPLC-grade water with 0.1% FA in acetonitrile ramped at a rate of 5.67 (v/v%) acetonitrile/min over 15 min. The flow rate was 0.7 mL/min. Sample volumes of 1 µL were injected. LL-37 standards, and samples were analyzed three times.

3.4.2 Data analysis

All HPLC-MS/MS LL-37 extraction samples and measurements were performed in triplicate and reported as mean ± SD.

3.5 Results and Discussion

Bond-Elut PPL, SOLA weak cation exchange (WCX), and C8 cartridges were tested to extract LL-37 from 10 ppm spiked standards. Bond-Elut PPL has been used in the laboratory previously with a wide range of hydrophobic to hydrophilic compounds and LL-37 is known to be hydrophilic [186, 187]. SOLA WCX was used to test an ion-exchange extraction due to LL-37's positive charge at physiological pH (pI of 10.61) [187, 188]. Preliminary work in the lab involved the use of Bond-Elut PPL and SOLA WCX SPE cartridges. However, this data was not consistently reproducible. Previous work focused on an eluting solvent of 0.1% FA in ACN with both the Bond-Elut PPL and SOLA WCX column. While the Bond-Elut PPL cartridges resulted in no recovery, this eluting solvent with the SOLA WCX column showed recovery. This work started by attempting to determine how the samples were inconsistent in recovery.

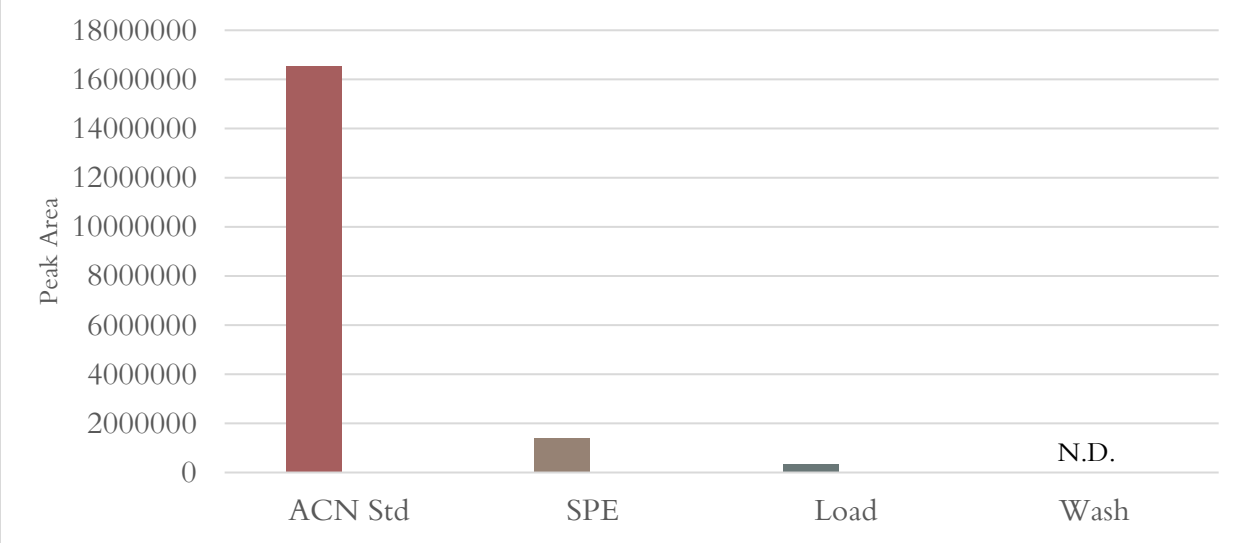


Figure 3.1 HPLC/MS data of 10 ppm LL-37 standards SOLA WCX SPE cartridges eluted in 0.1% FA in ACN. Wash and sample loading steps were analyzed to determine loss of sample during SPE procedures.

Figure 3.1 shows 10 ppm LL-37 standards eluted on a SOLA WCX SPE cartridge using 0.1% v/v FA in ACN. Both the loading and washing of the sample were analyzed to determine if sample was being lost in this process, and a schematic of the SPE process can be seen below. While some loss was observed, it does not account for the differences in standard and SPE peak area, leading to the conclusion that the sample was adhered to the column. Absolute recovery was calculated by taking the peak area from the standard and dividing by the peak area from the samples and multiplied by 100, as shown in Figure 3.1. Using this information, various elution solvents were used to determine if the sample could be eluted from the column.

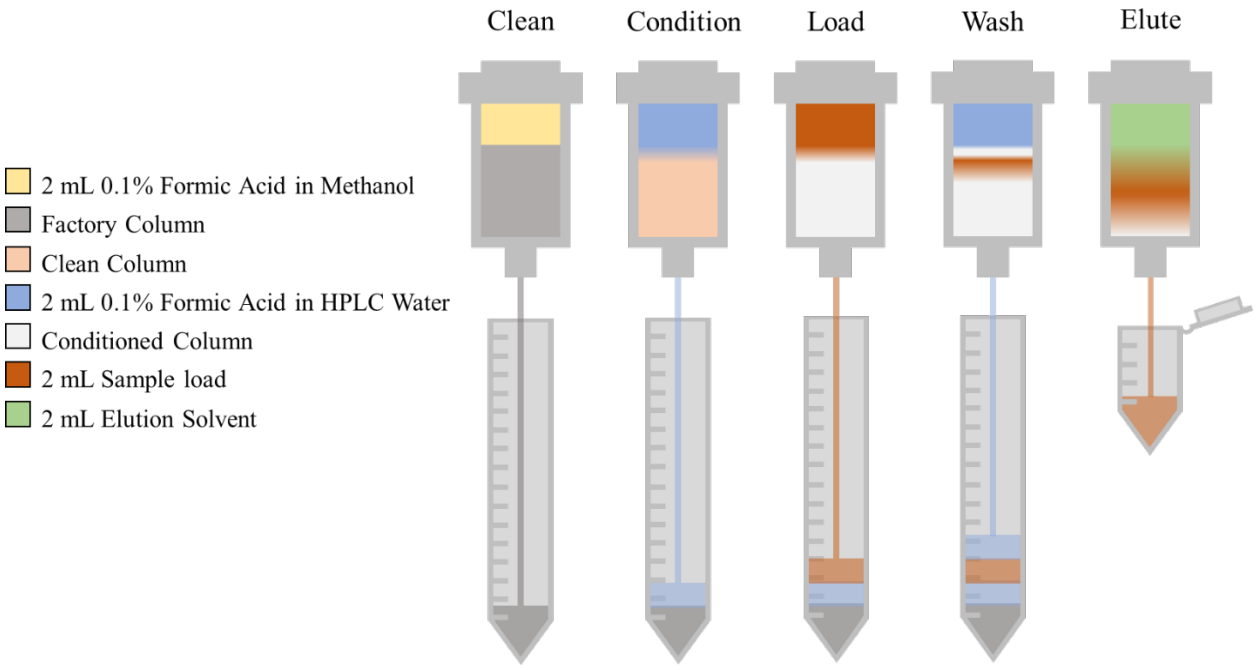


Figure 3.2 Flow diagram of SPE protocol

Figure 3.2 describes the solid-phase extraction procedures for all spiked-standards. For simplicity, Table 3.2 outlines the elution solvents explored in this work, labeled in green above.

Table 3-2 Elution solvents for solid-phase extraction columns.

	Solid-Phase Extraction	**Column**		
	Elution	SOLA WCX	Bond-Elut PPL	C8
1	0.1% FA in ACN	X	X**	X
2	1% FA in MeOH*	X		
3	5% FA in CAN	X	X	
4	5% FA in 60% CAN	X	X	X
5	**1.** 5% FA in ACN **2.** 2.5% DMSO in 60% ACN		X	
6	**1.** 5% FA in 60% ACN **2.** 50% DMSO in MeOH		X	
7	**1.** 5% FA in ACN **2.** 2.5% DMSO in MeOH	X		
8	**1.** 5% FA in 60% ACN **2.** 50% DMSO in 5% FA in 60% ACN	X		

* SOLA WCX Manufacturer's protocol, ** Previously established data

The SOLA manufacturer's protocol was also attempted to elute LL-37; however, no LL-37 was detected in either the load, wash, or elution. Procedures 3 and 4 as outlined in Table 3.1 were tested on both Bond-Elut PPL and SOLA WCX cartridges, and the loss due to either load or wash was analyzed.

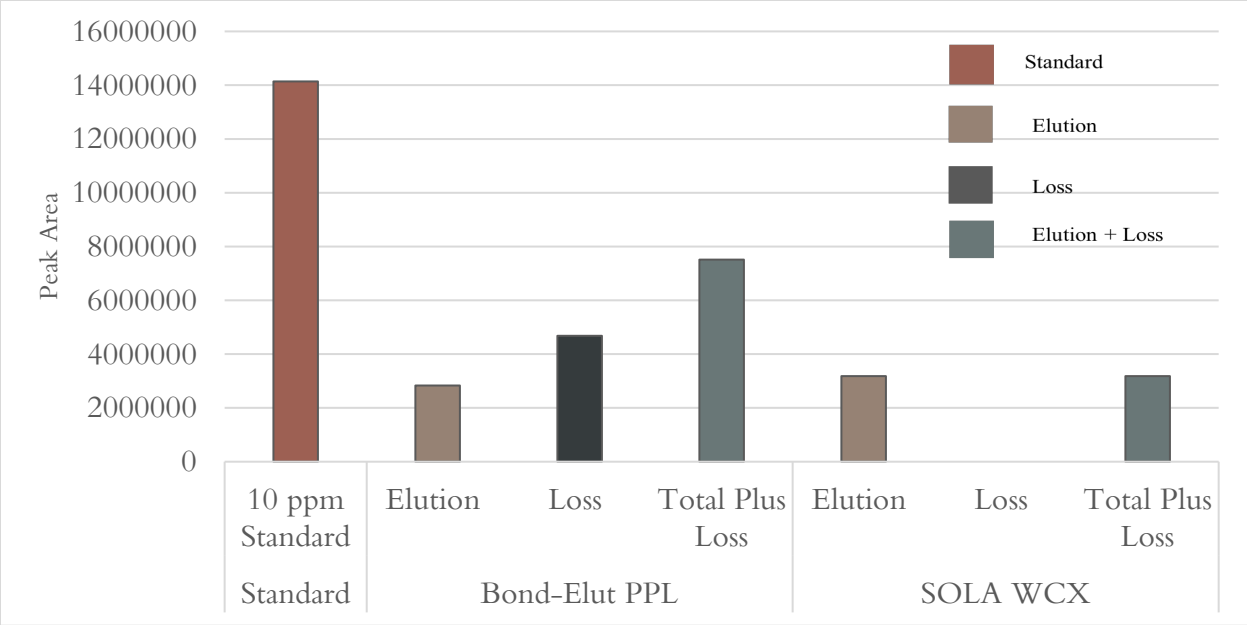


Figure 3.3 HPLC/MS data of SPE cartridges eluted in 5% FA in ACN. Wash and sample loading steps were analyzed to determine loss of sample during SPE procedures.

Figure 3.2 shows the results of direct injection following SPE elution in 5% v/v FA in ACN. It was proposed that a change in pH could alter the attachment of LL-37 to the cartridges. The use of the Bond-Elut PPL cartridges resulted in a larger loss than elution recovery, but still only accounts for approximately 53% of the sample. While there was no loss using the SOLA WCX cartridges, a recovery of 23%, was seen, leading to the conclusion that the standards are not being completely eluted.

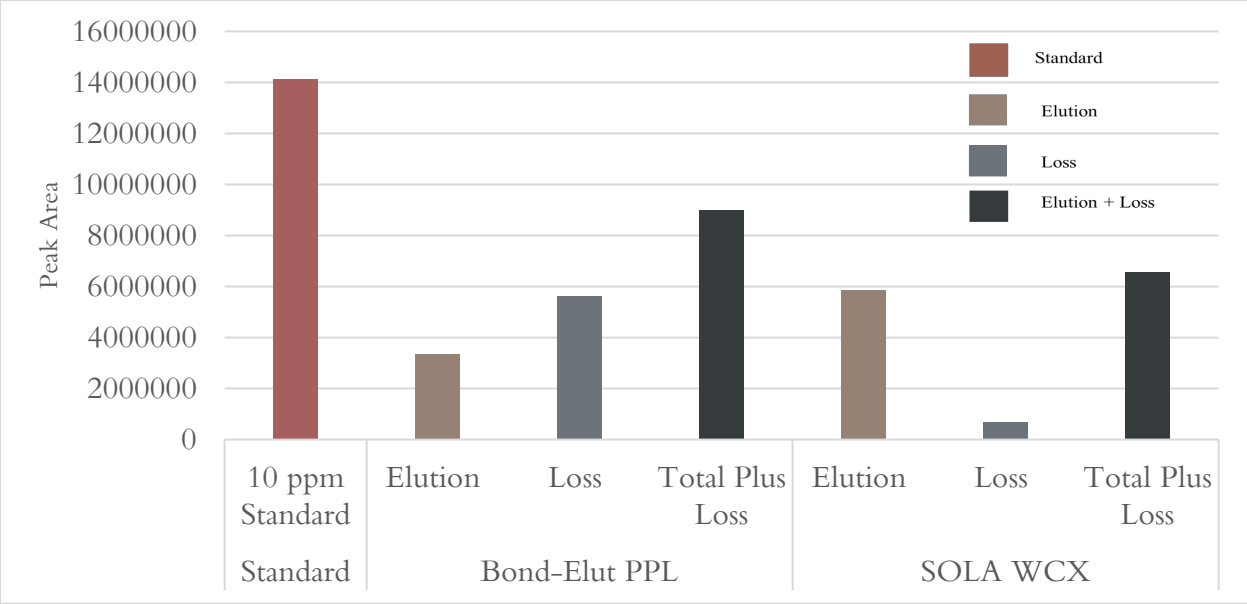


Figure 3.4 HPLC/MS data of SPE cartridges eluted in 5% FA in 60% ACN. Wash and sample loading steps were analyzed to determine loss of sample during SPE procedures.

As seen in Figure 3.3, Procedure 4 involved an elution phase of 5% FA in 60% ACN for both the Bond-Elut PPL and the SOLA WCX cartridges. This resulted in a similar effect as Procedure 3, where the Bond-Elut PPL cartridge led to more loss than elution. However, elution recovery was higher in both SPE cartridges, 23.8% and 43.5% for the Bond-Elut and SOLA, respectively. Using the results from Procedures 3 and 4, a method of double elution was proposed to get all of the sample out of the column. Because DMSO is used to clean the column between sample runs to remove residual analyte, it was chosen as a secondary elution solvent.

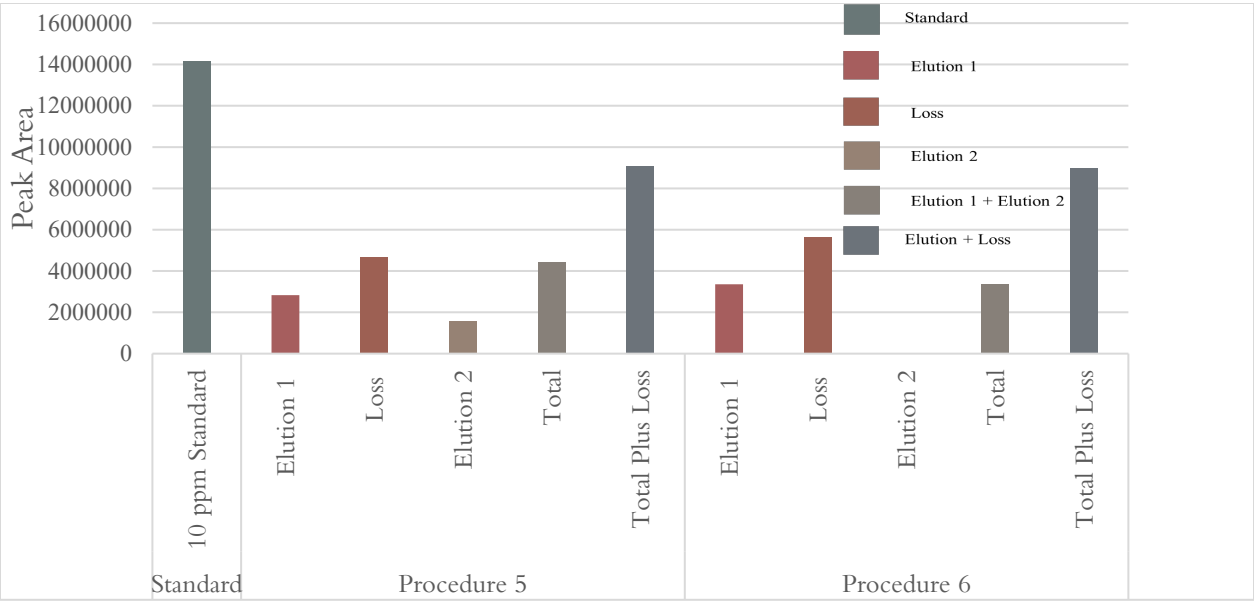


Figure 3.5 HPLC/MS data for Bond-Elut SPE cartridges following Procedures 5 and 6. Wash and sample loading steps were analyzed to determine loss of sample during SPE procedures.

Procedure 5 involves the use of a two-phase solid-phase extraction method; first, Bond-Elut PPL cartridges were used with 5% FA in ACN as an eluting solvent. Then, 2.5% DMSO in 60% ACN was used as a secondary elution phase. While the second elution did remove more sample from the cartridge, recovery was only at 31.2%, or 64.4% when accounting for loss, as ACN be seen in Figure 3.4. Procedure 6 outlines a first elution phase of 5% FA in 60% ACN, followed by a second elution of 50% DMSO in MeOH, using the Bond-Elut PPL cartridge. It was proposed that increasing the percentage of DMSO could result in a higher elution. This paired with the standard elution solvent of methanol resulted in no recovery for the second elution phase. Due to the higher recovery and lower loss of analyte, SOLA WCX cartridges were explored with a double elution, as seen in Figure 3.5.

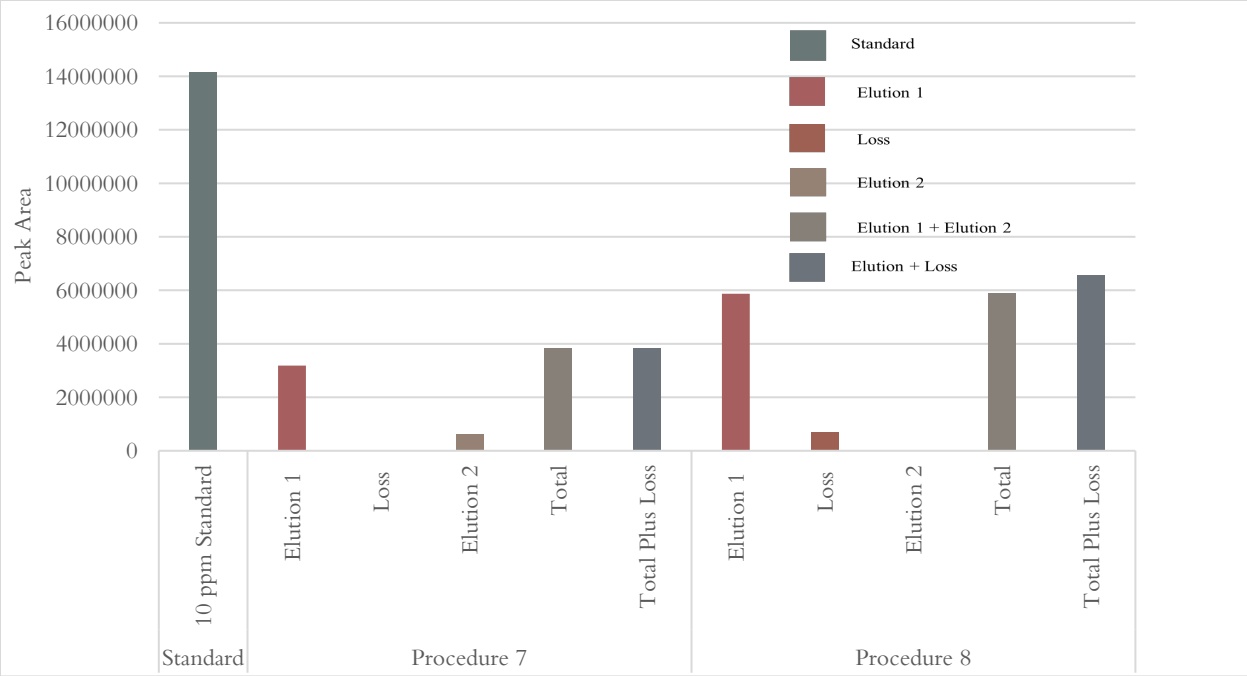


Figure 3.6 HPLC/MS data for SOLA WCX SPE cartridges following Procedures 7 and 8. Wash and sample loading steps were analyzed to determine loss of sample during SPE procedures.

Procedure 7 follows a first elution step of 5% FA in ACN and 2.5% DMSO in MeOH as a second elution solvent. Secondary elution with 2.5 % DMSO in methanol resulted in an increase of recovery from 22.5% to 26.9%, with no loss being observed in the wash or load phases. Procedure 8 outlines a first elution phase of 5%FA in 60% ACN followed by elution with 50% DMSO in 5% FA in 60% ACN. Similar to Procedure 6, the 50% DMSO in varying solvents resulted in no recovery for the second elution phase. Upon exhausting a multitude of elution solvents for both SOLA WCX and Bond-Elut PPL SPE cartridges, it was determined that the C18 modified phases were not suitable for LL-37 extraction. C8 SPE cartridges with a 50 mg bed capacity were explored as a possible solution.

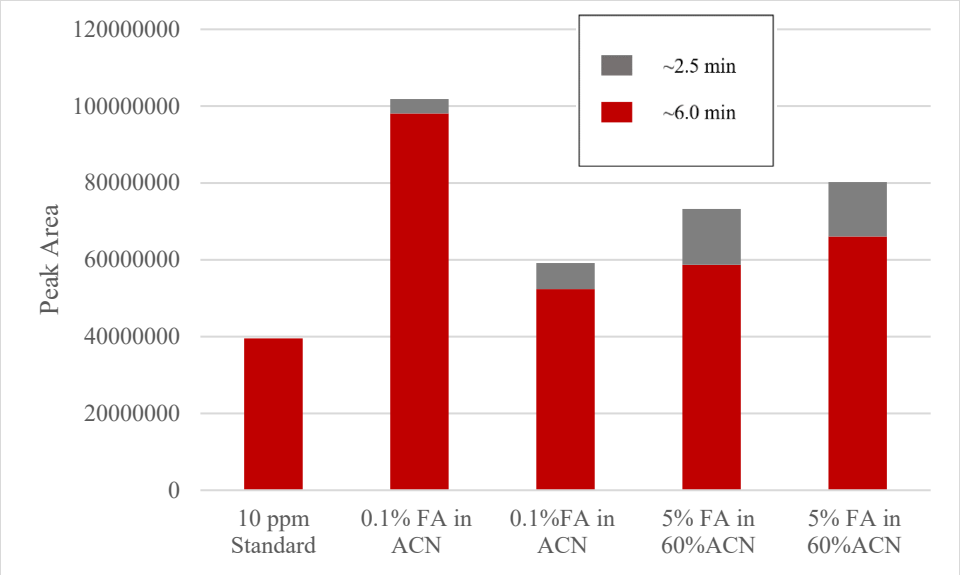


Figure 3.7 HPLC/MS data for C8 SPE cartridges using 0.1% FA in ACN or 5%FA in 60% ACN as an elution solvent.

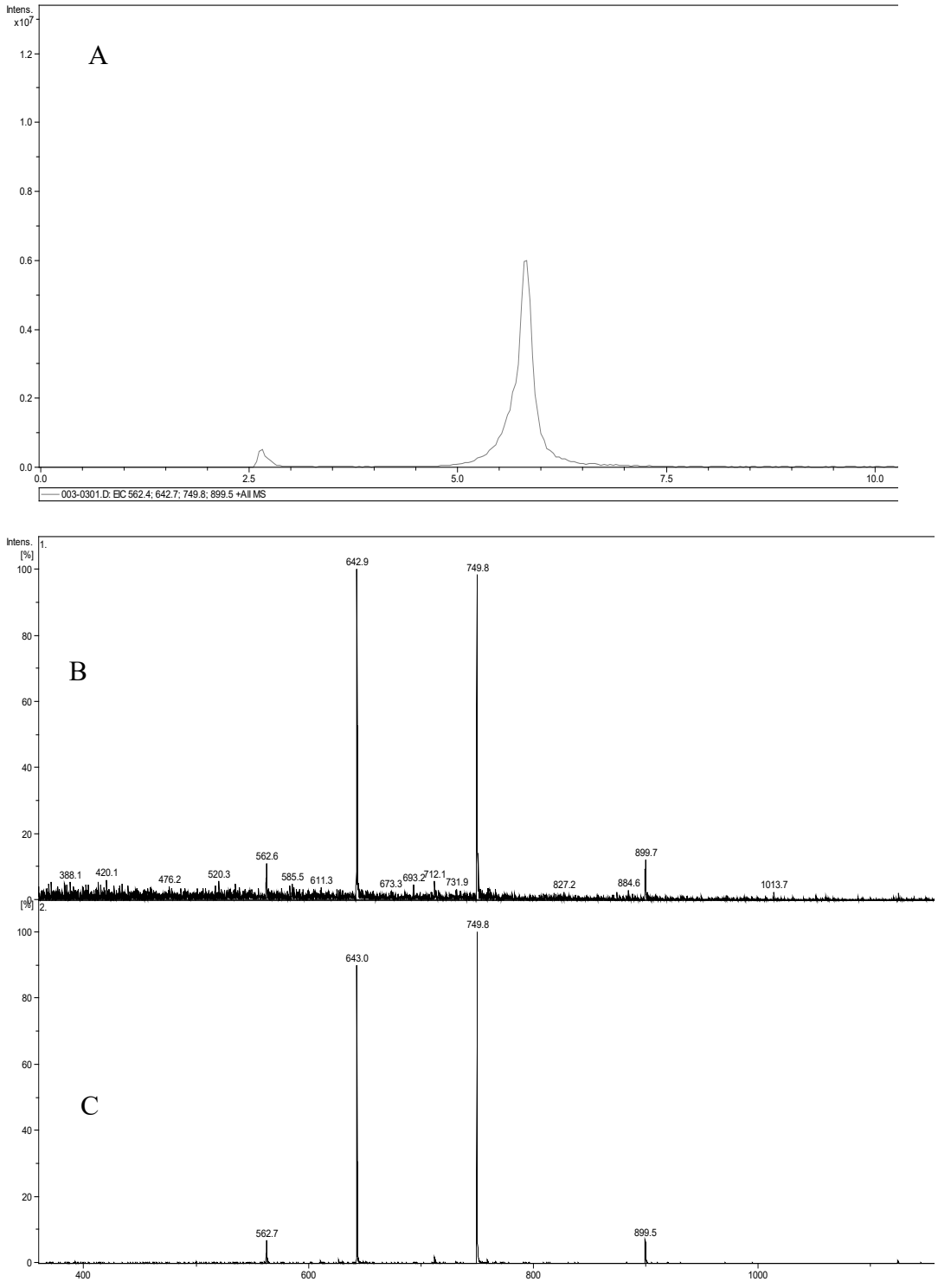


Figure 3.8 (A) EIC for C8 SPE cartridges using 0.1% FA in ACN or 5% FA in 60% ACN as an elution solvent. Peak 1 occurs at 2.7minutes, and peak 2 at 5.7 minutes. Mass spectra for peak 1 (B) and peak 2 (C).

Using the two most promising elution solvents of 0.1% FA in ACN or 5% FA in 60% ACN, C8 SPE cartridges were evaluated to extract LL-37. Figure 3.7 shows that both solvents were effective in extracting LL-37, with average recoveries of 203.7% and 194.1%, respectively. However, these processes resulted in two peaks with identical m/z ratios, one at 2.7 minutes and another at 5.7 minutes, as can be seen in Figure 3.8. The LL-37 (10ppm) standard resulted in a single peak at 5.7 minutes. In an attempt to merge the two peaks, and have them co-elute, the solvent ramp was changed. Initially, a linear gradient composed of 0.1% formic acid (FA) in HPLC-grade water with 0.1% FA in acetonitrile ramped at a rate of 7.5 (v/v%) acetonitrile/min over 10 min, with a flow rate was 0.3 mL/min was used. This was adjusted to a linear gradient composed of 0.1% formic acid (FA) in HPLC-grade water with 0.1% FA in acetonitrile ramped at a rate of 5.67 (v/v%) acetonitrile/min over 15 min, with a flow rate of 0.7 mL/min. Additionally, the recovery greater than 100% in both samples suggested that proper analysis needed to be done at lower concentrations so as not to hit the solubility limit, as previously discussed in Chapter 2. Due to the limitations of the Bruker-Daltonics HPLC/MS, the HPLC/MS/MS from Shimadzu was used.

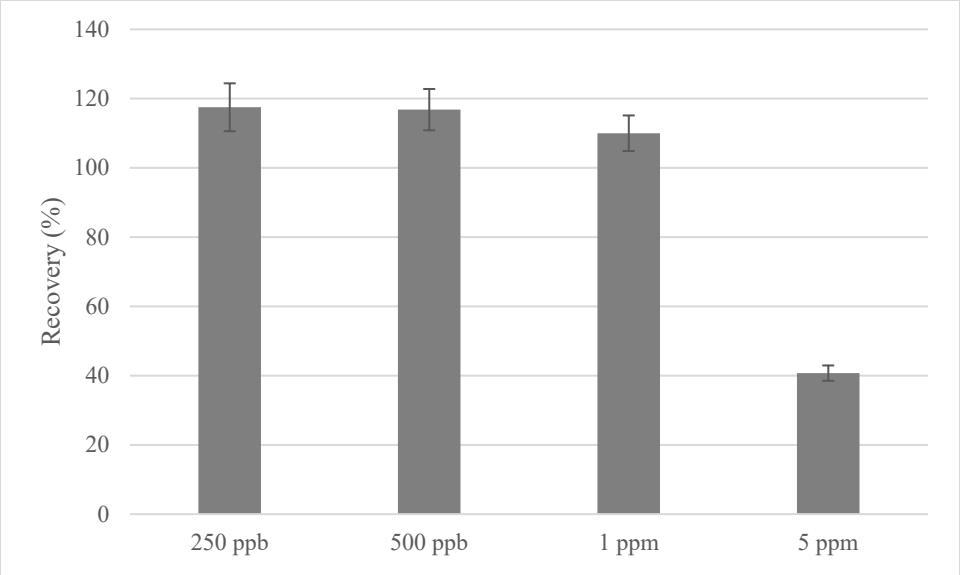


Figure 3.9 HPLC/MS/MS data for LL-37 C8 SPE cartridges using 0.1%FA in ACN (n=4).

Due to the higher recovery of LL-37 samples using an elution of 0.1% FA in ACN, it was used as the elution solvent for C8 SPE analysis on the HPLC/MS/MS. Using direct comparison of LL-37 standards and solid-phase extraction samples, it was determined that C8 cartridges may be viable (Figure 3.9), however, as the calibration curve could not be used to determine concentration, it was determined that higher concentrations of LL-37 (5 ppm and above) are not feasible for this method. As preliminary data showed that for a 24-co-culture of *Staphylococcus aureus*, a concentration of less than 1 ppm is to be expected, this method could prove viable. However, the new solvent ramp was successful in co-elution of the two peaks with duplicate m/z ratios, as only one peak with a retention time of 6.5 minutes was observed. As Chapter 2 showed, time plays a role in LL-37 consistency. Using the C8 SPE cartridges, samples were studied over the course of 72 hours. For this preliminary work, the Bruker-Daltonics HPLC/MS was used to keep experimental costs down.

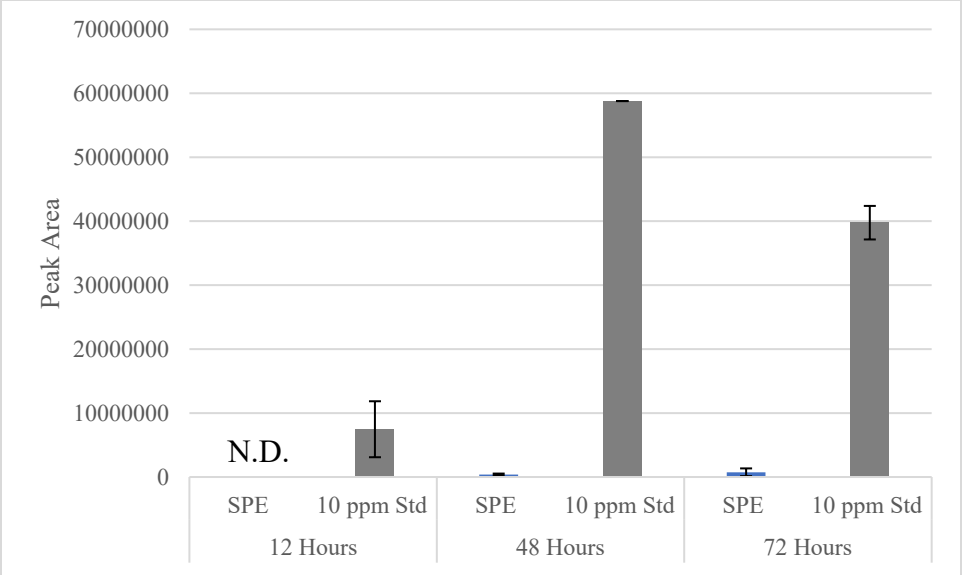


Figure 3.10 HPLC/MS data for C8 SPE cartridges over time, after reconstitution in water (n=3).

Figure 3.10 shows LL-37 samples eluted with 0.1% FA in ACN using a C8 cartridge. Samples were dried and reconstituted in water, as biological samples will have to undergo drying phases. However, results over time showed no recovery after 12 hours, and only a recovery of 1.9 % after 72 hours at room temperature. Given the low percent recovery in relation to previous sample analysis, another direct injection experiment for LL-37 was run over 72 hours, the results of which can be seen in Figure 3.11.

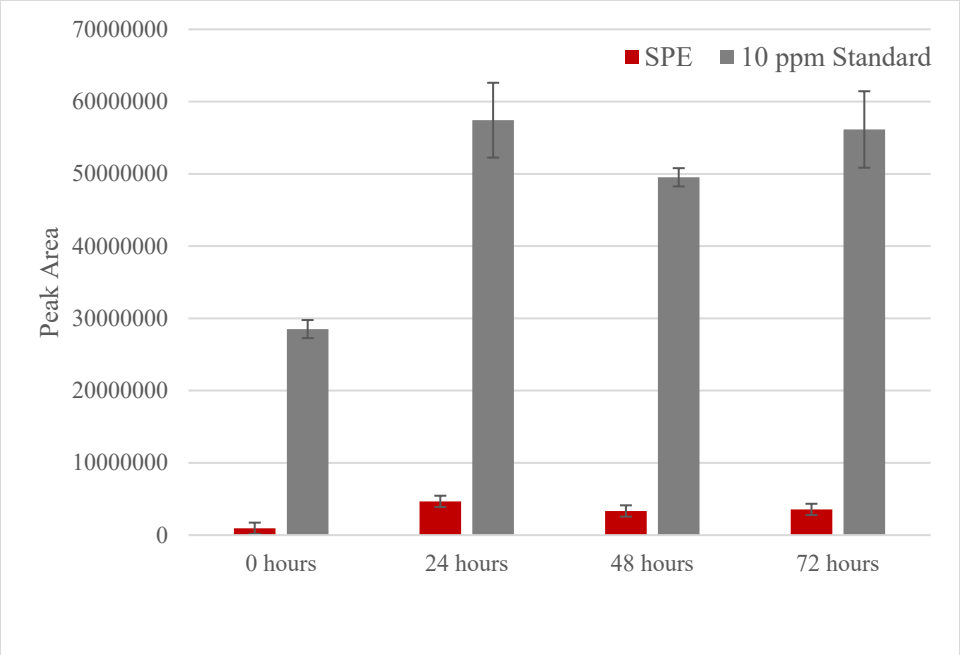


Figure 3.11 HPLC/MS data for C8 SPE cartridges over time (n=3).

While recovery is higher than for the reconstituted LL-37 samples seen in Figure 3.10, it is much lower than previous studies, as seen in Figure 3.7 and Figure 3.9. It was determined that the cause may be due to a change of vendors that occurred due to a backorder of supply from the COVID-19 pandemic. Figure 3.10 and Figure 3.11 were using Sigma-Aldrich LL-37, whereas Figure 3.7 and Figure 3.9 came from Bachem LL-37. To test this, BACHEM LL-37 was reacquired and evaluated using direct injection following C8 solid-phase extraction, as can be seen in Figure 3.12.

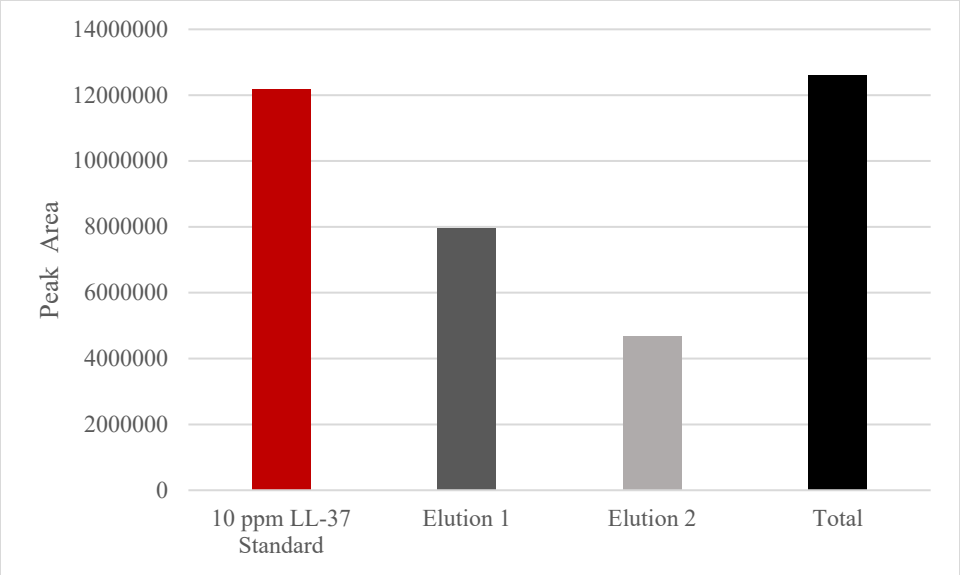


Figure 3.12 HPLC/MS data for Bachem LL-37 (10ppm) using a two-phase elution.

Bachem LL-37 was analyzed at timepoint 0, similar to Figure 3.11, which uses Sigma-Aldrich LL-37, to compare the two vendors, as seen in Figure 3.10. Elution 1 involved the same elution solvent from Figure 3.11, 0.1% FA in ACN, and resulted in a recovery of 65.3%. From this promising result, a second elution phase of 5% FA in 60% ACN, also the preferred solvent from Chapter 2 was used. This resulted in an additional recovery of 38.4%, or a total recovery of 103.7%. From this, it was determined that a single elution of 5% FA in 60% ACN could result in sample recovery, the results of which can be seen in Figure 3.13.

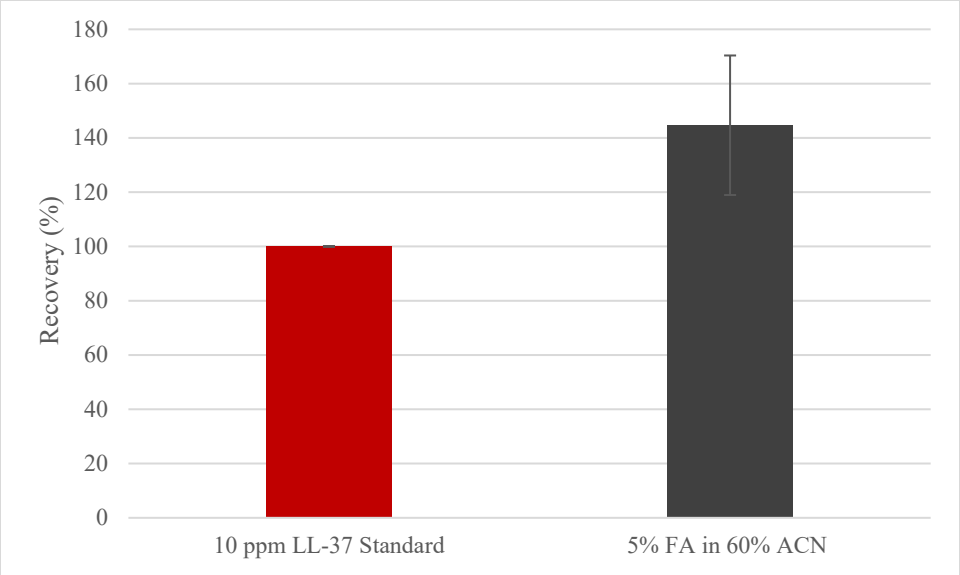


Figure 3.13 HPLC/MS data for Bachem LL-37 using 5% FA in 60% ACN (n=4).

Using Bachem LL-37, Figure 3.13 shows an average percent recovery of 144.7%. This recovery greater than 100% was expected as this was performed at 10 ppm on the Bruker-Daltonics HPLC/MS, as discussed in Chapter 2. To ensure viability of the method, this solid-phase extraction method was performed on the Shimadzu HPLC/MS/MS with appropriate concentrations within the solubility limit.

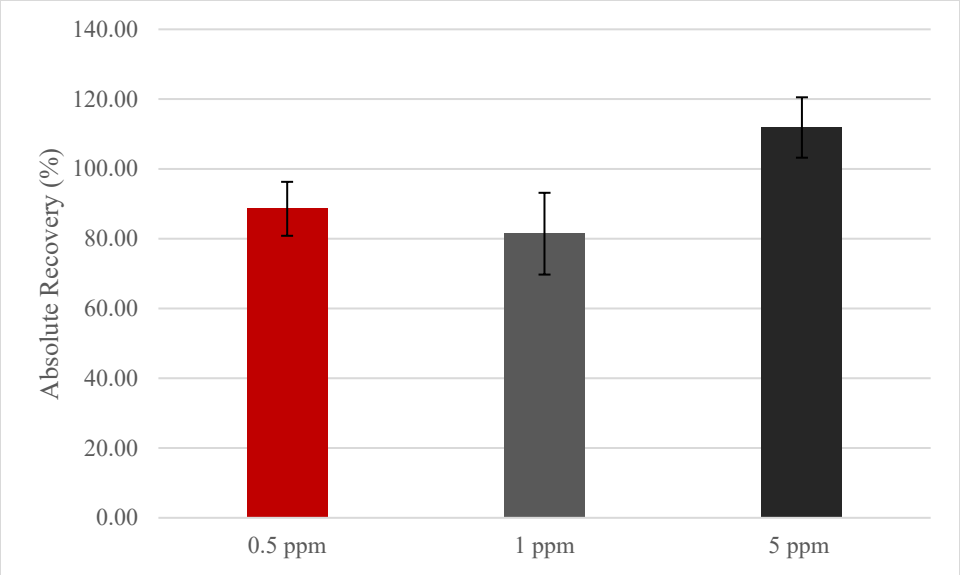


Figure 3.14 HPLC/MS/MS data for Bachem LL-37 using C8 SPE cartridges with 5% FA in 60% ACN (n=4).

Using the new Bachem LL-37, three concentrations were chosen (5 ppm, 1ppm, and 0.5 ppm) to validate the solid-phase extraction method for LL-37 (Figure 3.14). While 5 ppm has breached the solubility limit slightly, with a recovery of 111.9%, both 1 ppm and 0.5 ppm LL-37 SPE's result in appropriate recoveries of 81.43 ± 11.7% and 88.56 ± 7.73%, respectively, where n=4. The limit of detection (LOD) was found to be 0.496 ppb and the limit of quantitation (LOQ) was found to be 1.65 ppb. Moving forward, only Bachem LL-37 will be used for analysis.

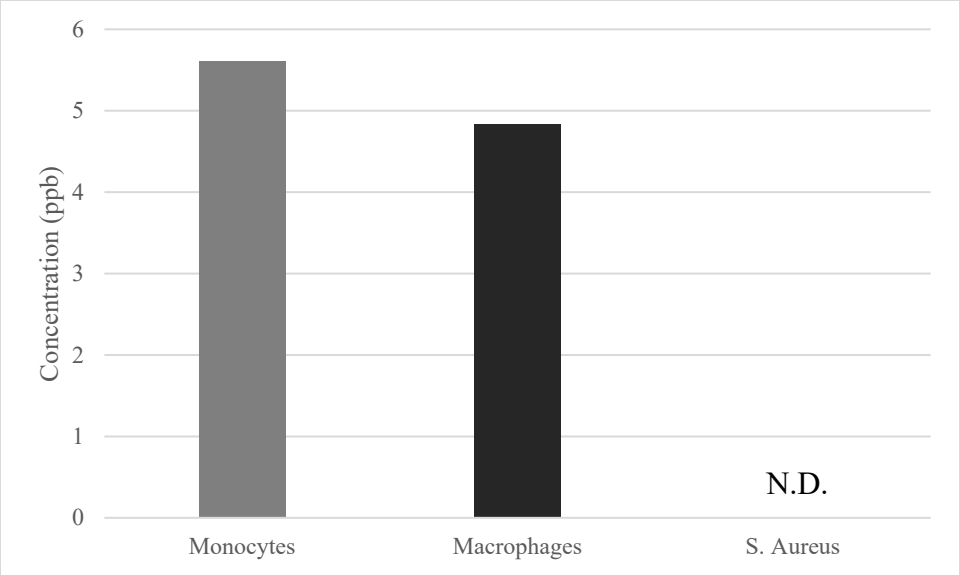


Figure 3.15 Preliminary HPLC/MS/MS data for the detection of LL-37 in biological species. Endogenous levels of LL-37 were analyzed for monocytes, macrophages, and S. aureus biofilms.

In order to move on to Chapter 4, the method established in Figure 3.13and Figure 3.14 needed to be tested on a biological sample. Here, monocytes and macrophages at a concentration of 1 x 10^6 cells/mL in cell media underwent solid-phase extraction and HPLC-MS/MS analysis, Figure 3.15. Staphylococcus aureus biofilms were made and analyzed as well, however, baseline levels were below the limit of quantitation. As this demonstrates preliminary data, all biological samples were prepared according to methods outlined in Chapter 4.

3.6 Conclusion

The purpose of this work was to determine a method to extract LL-37 from spiked standards using solid-phase extraction. Bond-Elut PPL cartridges were tested due to its ability to retain hydrophilic analytes, whereas SOLA WCX was explored for its use of an ion-exchange cartridge, both of which are proprietarily modified C18 phases. C8 SPE cartridges were explored due to the possibility of aggregation of samples blocking full elution in the C18 phases. Using an

elution phase of 5% FA in 60% ACN, which was also the preferred solvent for sample consistency found in Chapter 2, LL-37 spiked standards were determined to have an average absolute recovery of 85.0%. Furthermore, LOD was found to be 0.496 ppb, and LOQ 1.65 ppb. This method will be used in Chapter 4 to determine biological concentrations of co-cultures involving macrophages and bacterial biofilms.

4 Analysis of LL-37 in *in vitro* co-cultures of activated THP-1 cells and bacterial biofilms

4.1 Introduction

According to the Centers for Disease Control (US), approximately 1.7 million hospitalized patients acquire a healthcare-associated infection each year, resulting in over 98,000 deaths and anywhere from $28.4 to $45 billion in treatment costs [2]. Over the years there is a rise of antimicrobial-resistant bacteria in the medical community has become a major problem in the treatment and control of bacterial infection. A new and novel therapeutic strategy for the control and treatment of a bacterial infection that involves the reduction of antibiotic resistance organisms has been shown to be important. *Pseudomonas aeruginosa (P. aeruginosa)* has been isolated in many wounds and infection states, such as meningitis, urinary tract infections, respiratory systems, sepsis in infants, and others [1, 3]. *Staphylococcus aureus (S. aureus)* is one of the most commonly isolated microorganisms and is frequently found in both infant wards and ICUs [5, 6]. In an effort to avoid antibiotics, the study of new therapeutic anti-microbial agents has been developed. Antimicrobial peptides (AMPs) have shown to be a promising alternative to conventional antibiotics.

AMPs are known to be antimicrobial agents that kill the bacteria via membrane disruption, and they have been shown to have broad-spectrum activity and immunomodulator[109]. As an immunomodulator, the precursor LL-37 (hCAP-18 genes) has shown in the synthesis and secretion of this peptide by the epithelial cells. In addition, immune cells such as macrophages, dendritic cells, natural killer cells, neutrophils, and mesenchymal stem cells have been shown in other studies that secrete LL-37 in humans [121, 122]. Moreover, LL-37 is known to play a role in the use of chemoattracts to modulate the production of various inflammatory factors [148].

In this chapter, LL-37 levels were analyzed using the newly developed method in an *in vitro* setting: the *in vitro* method consisted of THP-1 activated co-culture with bacterial biofilms of *S. aureus, S. epidermidis,* and *P. aeruginosa*. Given the importance of bacterial biofilms, due to their prevalence in HCAIs, as well as their increased antibiotic resistance that can be observed in comparison to their planktonic counterparts, they were the primary focus of this work. [3, 4, 14, 30]. Furthermore, the study of biofilm interaction not only between bacterial species, but also, interactions within the host, can be analyzed through the use of an *in vitro* co-culture system. This chapter was focused on the co-culture of macrophages and bacteria-biofilm interaction and the possibility of the use of LL-37 as a therapeutic treatment in chronic infections and wounds. Using a co-culture, and the sample preparation techniques established in Chapters 2 and 3, LL-37 is able to be sampled, extracted, and measured using HPLC-MS/MS. It was proposed that analyzing the concentration of LL-37 over time during an infection-state, could provide valuable information on how LL-37 interacts within the host, which could lead to potential therapeutic applications.

4.2 Materials and methods

4.2.1 Materials and reagents

Staphylococcus epidermidis (35984), *Staphylococcus aureus* (25923), and *Pseudomonas aeruginosa* (700829) were purchased from ATCC. Tryptic soy broth (TS), brain heart infusion broth (BHI), glycerol, sodium chloride, glucose, and agar were purchased from VWR. Fibrinogen (Fb) fraction 1 (from bovine plasma) and bovine serum albumin were purchased from VWR International (Radnor, PA). Polycarbonate was obtained from Gizmo Dorks (Temple City, CA). THP-1 cells were a gift to the Stenken lab from Dr. Steven Wood from the FDA. Roswell Park Memorial Institute (RPMI-1640) medium and Hank's solutions were obtained from ATCC

(Manassas, VA) and VWR (Radnor, PA). Fetal bovine serum (FBS), antibiotic-antimycotic solution (100x), and phorbol 12-myristate 13-acetate (PMA) were all obtained from Sigma-Aldrich (St. Louis, MO). The lipopolysaccharide (Salmonella typhimurium, LPS) was obtained from Calbiochem (Massachusetts, MA). High-performance liquid chromatography (HPLC)-grade water, formic acid, and acetonitrile were obtained from Fisher Scientific (Hanover Park, IL). C8 SPE cartridges with a 50 mg bed capacity were obtained from Thermo Fisher Scientific (Waltham, MA). LL-37 was obtained from Bachem (Torrance, CA).

4.2.2 Spread plate preparation

Cultures were stored in 30% glycerol at -80 °C and streak plated prior to experimental preparation. From frozen stock, cultures were prepared by inoculation of a single colony into disposable culture tubes overnight containing either 5 mL BHI (*P. aeruginosa and S. aureus)* or TS (*S. epidermidis)*, that had been filtered using a 0.45 um syringe filter. Bacteria were then counted using optical density at 600 nm (O.D. 600), to determine CFU/mL, and diluted to a final concentration of ~1 x 10^3 CFU/mL using 1x PBS. Bacteria were then streak plated on either tryptic soy or brain heart infusion agar plates, and incubated overnight at 37 °C.

4.2.3 Biofilm formation for S. epidermidis, S. aureus, and P. aeruginosa

Prior to biofilm formation, Patrick Pysz, M.S. used 1.75 mm clear polycarbonate to design and fabricate 3D-printed growth lattices that were used as a structural support system, to promote biofilm attachment, which have been previously used in our lab, which can be seen in Figure 4.1 (B) below.[1] To promote biofilm formation, the culture medium was supplemented with 7 mg/mL of fibrinogen, with 1% (w/v) of sodium chloride, 3% (w/v) of bovine serum albumin, and 1% (w/v)

[1] Attachment and optimization of *Staphylococcus aureus*, *Staphylococcus epidermidis,* and *Pseudomonas aeruginosa* biofilms to 3D printed lattice allowing facile transfer into a macrophage-cell culture. Paper submitted and under review.

of glucose, and filtered (0.45 um) to maintain sterility. Using the previously prepared spread plates, 5 mL of medium was inoculated with a single colony, and an autoclaved growth lattice was added, before incubation at 37 ºC at 170 RPM (Benchtop shaker). Using previously developed methods, each bacterial biofilm was incubated until an OD 600 reading of 0.3-0.5 absorbance units. For *S. aureus,* this growth time is 21 hours; for *S. epidermidis,* 14 hours; and for *P. aeruginosa,* 13 hours. To verify biofilm growth size, a biofilm was randomly chosen, and the growth lattice-biofilm was transferred to a conical tube containing 5 mL of 1x PBS and placed in a water bath for 10 mins at 37 °C. The solution was then vortexed and the OD600 was measured. [221]

4.2.4 Activated macrophage-like cells from THP-1 cells

THP-1 cells were grown using 89% V/V RPMI-1640 medium, 10% V/V FBS and 1% V/V antibiotic-antimycotic solution. Monocyte counts were performed using a hemocytometer containing a 1:1 ratio of trypan blue and cell culture medium, and 1.0 x10 ^{6} cells/mL were added to each well or flask. Monocytes were converted to macrophage-like cells using 10 μM of PMA and incubated for 24 hours at 37 °C in 5% CO_2. Characterization of macrophages occurred by observing the attachment of cells to the well surface, then washing with Hank's solution to rid the culture of any planktonic monocyte remainders. Subsequent to washing, a cell medium free of antimycotic-antibiotic was added.

4.2.5 Co-culture of macrophages with bacterial biofilms

For the co-culture of macrophage-like cells and bacteria-biofilm, bacterial biofilms of *S. epidermidis, S.aureus,* and *P. aeruginosa* were placed into macrophage-like cell cultures and incubated for 24 (*P. aeruginosa*) or 48 hours at 37 °C in 5% CO_2. The supernatant of the bacterial macrophage co-culture was collected following centrifugation at 1200 RPM (337 xg RCF) for 5 min and then frozen at -80 °C until needed.

4.2.6 Sample preparation and analysis

4.2.6.1 Sample preparation using solid-phase extraction (C8-SPE)

Using Chapter 3's verified method, C8 SPE cartridges (Agilent C8) were washed with 2 mL methanol with 0.1 % v/v formic acid. Then, the column was pre-conditioned with 2 mL of HPLC grade water with 0.1 % v/v formic acid, before 2 mL of sample was added to the SPE column. The column was washed with 2 mL of HPLC grade water with 0.1% v/v formic and the sample was eluted with 2 mL of 5% FA in 60% (v/v) ACN. Samples were then dried using a CentriVap Vacuum and reconstituted to 200 µL using 5% FA in 60% (v/v) ACN.

4.2.6.2 HPLC-MS/MS analysis

HPLC-MS/MS analysis was performed using a Shimadzu UPLC-20A/HPLC-30A in line with a Shimadzu 8060 triple quadrupole mass spectrometer with a heated electrospray source (positive-ion mode). Separations were performed using a C8 column (4.6 × 150 mm, 5 µm particle size, SUPELCO, Discovery BIO Wide Pore) with a linear gradient composed of 0.1% formic acid (FA) in HPLC-grade water with 0.1% FA in acetonitrile ramped at a rate of 5.67 (v/v%) acetonitrile/min over 15 min. The flow rate was 0.7 mL/min. Sample volumes of 1 µL were injected.

4.2.7 Data analysis

All measurements were performed in quadruplicate (n=4) and reported as mean ± SD. A student's T-test was used to determine statistical significance.

4.3 Results and discussion

4.3.1 Co-culture of bacteria-biofilm and macrophages-like cells

As Chapter 3 developed a sample preparation method for biological sampling, Chapter 4 is the culmination of these techniques for use in an *in vitro* co-culture for the detection and

quantification of LL-37. To do this, co-cultures comprised of activated THP-1 cells (macrophage-like cells) and bacterial biofilms were incubated and sampled every 12 hours for 24 or 48 hours (Figure 4.1 (C)). The cultures were checked to ensure macrophage viability through the color change of the cell medium. This is because a color change from orange-pink to yellow indicates that the macrophages are no longer viable, as can be seen in Figure 4.1 (A) below.

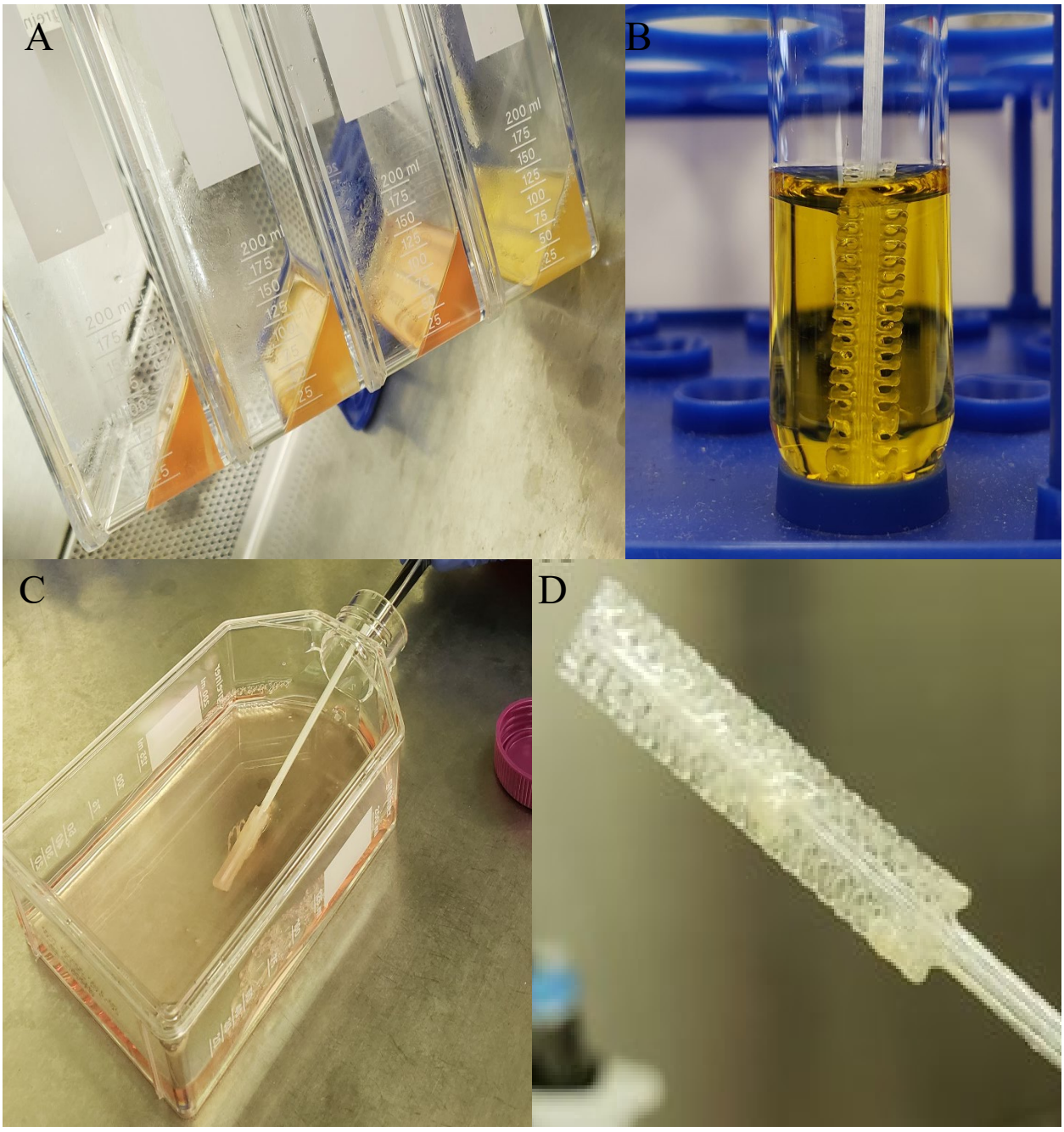


Figure 4.1 (A) Co-culture medium colors; right-most is not viable. (B) 3D printed toothbrush in bacteria media, (C) Observable biofilm on toothbrush introduced to macrophage-like cells in culture flask. (D) Observable biofilm (*S.aureus*) on 3D-printed toothbrush.

Previous work in the laboratory has shown that it is important to promote biofilm attachment using supplements in the bacteria culture such as 7 mg/mL fibrinogen, 3% w/v of BSA, 1% w/v of sodium chloride, and 1% w/v of glucose [149]. 3D printed biofilm growth lattices and supplements were used to promote biofilm growth and attachment, easing transfer between culture tubes, and co-culture flasks. An observable biofilm is shown on a growth lattice in Figure 4.1 (A). After the co-culture of macrophages-like cells and bacteria-biofilm, the samples were collected every 12 hours for 48 hours. After the collection, samples were run through solid-phase extraction cartridges (SPE), prior to HPLC-MS/MS analysis.

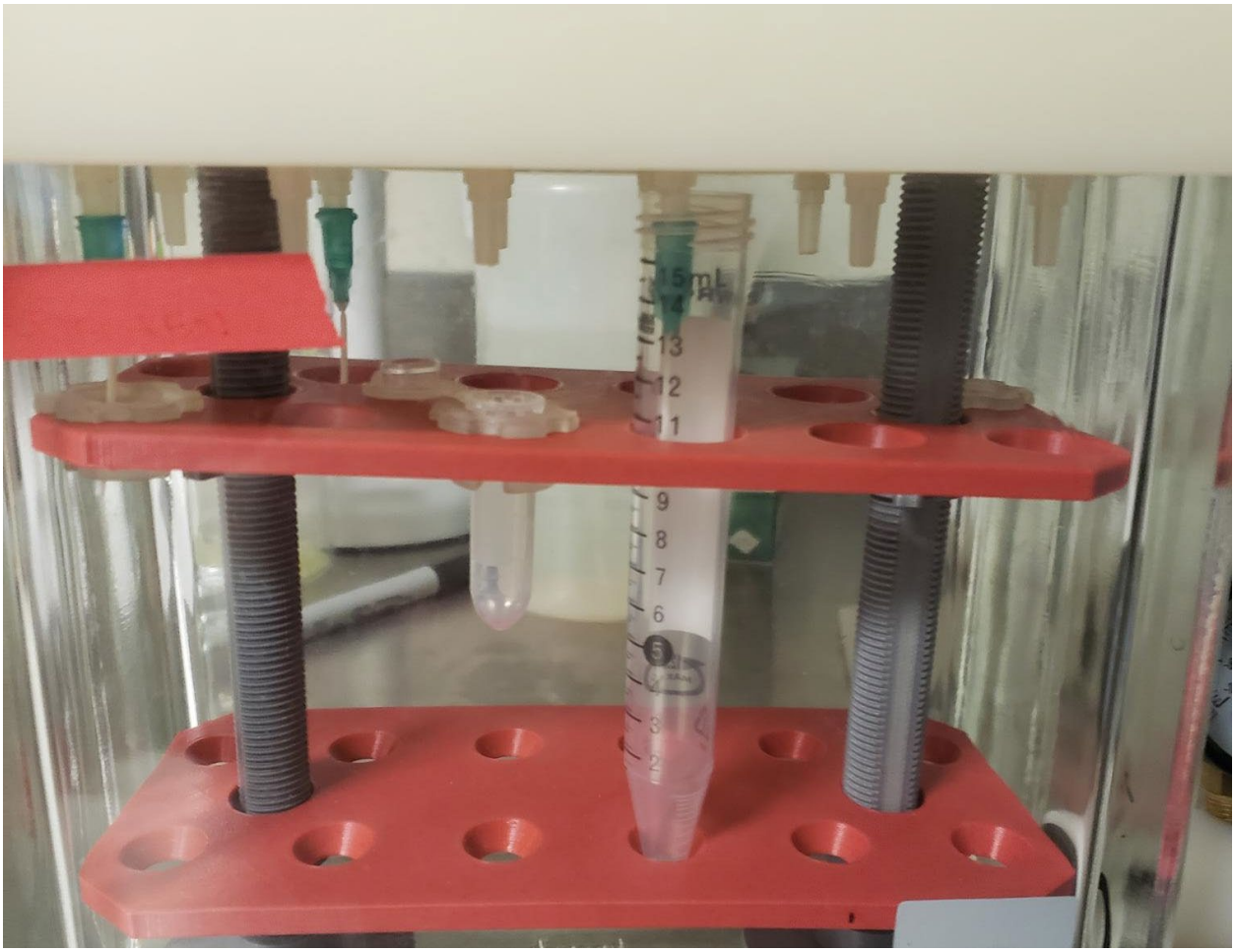


Figure 4.2 3D printed SPE collection chamber.

Preliminary data showed that LL-37 was adsorbing to the plastic of standard microcentrifuge tubes during drying and reconstitution. To counteract this, Patrick M. Pysz, M.Sc. was recruited to fabricate a modified solid-phase extraction collection chamber, allowing for the interchange between wash collection and protein lo-bind tubes for elution. This straightforward design allowed for the same tubes to be used for elution and reconstitution, decreasing potential sample loss (Figure 4.2).

4.3.1.1 Co-culture of *Staphylococcus aureus*-biofilm and macrophage-like cells

Staphylococcus aureus-biofilms, Figure 4.1 (D), were co-cultured with activated THP-1 cells for 48 hours, and levels of LL-37 were measured using HPLC-MS/MS. Macrophage controls for all biofilm co-cultures showed non-detectable levels of LL-37, as Figure 4.2 showed. In addition to control, macrophages were introduced to a biofilm of *S. aureus*, and elevated levels of LL-37 can be seen at 12 and 24 hours, with average concentrations of 7658 ± 2455 pg/mL and 1005 ± 264 pg/mL, respectively (Figure 4.3). One important note in Figure 4.3 is the *S. aureus*-biofilm co-culture showed the highest secretion of LL-37 at 12 hours of incubation compared with other bacterial co-cultures. Additionally, despite the co-culture-maintaining viability beyond 48 hours, both time points of 36 and 48 hours showed non-detectable levels of LL-37.

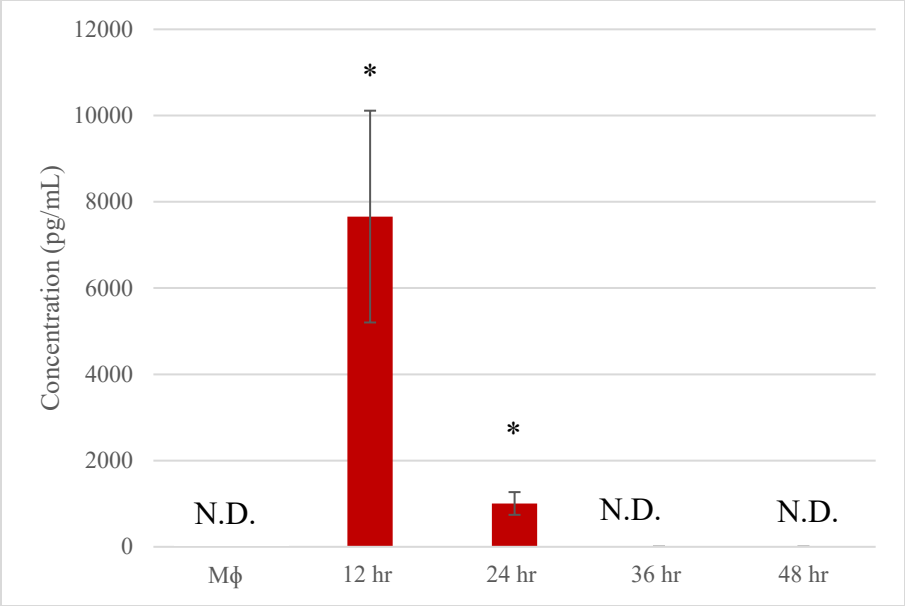


Figure 4.3 Concentration of LL-37 (pg/mL) during the co-culture of *S. aureus* biofilm (4.0 x 10^8 CFU/mL) with activated THP-1 (macrophage-like cells) (1.0 x 10^6) cells/mL. Aliquots 3 mL of the co-culture were extracted every 12 hours for 48 hours. After collection, the sample was prepared using SPE (C8) and analyzed using HPLC-MS/MS. Error bars denote mean ± SD, N=4 for each time point, and significantly different time points from the control where denoted by * $p<0.05$

4.3.1.2 Co-culture of *Staphylococcus epidermidis*-biofilm and macrophage-like cells

Staphylococcus epidermidis, another Gram-positive bacterium was co-cultured with macrophage-like cells over 48 hours as well. However, while it is possible to see LL-37 at 12 and 24 hours, before becoming non-detectable at 36 and 48 hours in *S. aureus*, there is a delayed effect in the co-culture of *S. epidermidis*-biofilm. Here, LL-37 is not detectable until 36 hours, with an average concentration of 23 ± 14 pg/mL. Levels of LL-37 increase by a time point of 48 hours to an average concentration of 141 ± 70 pg/mL, as can be seen in Figure 4.4. An increase was observed after 36 hours of co-culture which could be a possibility that the "highest concentration of LL-37" was not observed in this experiment.

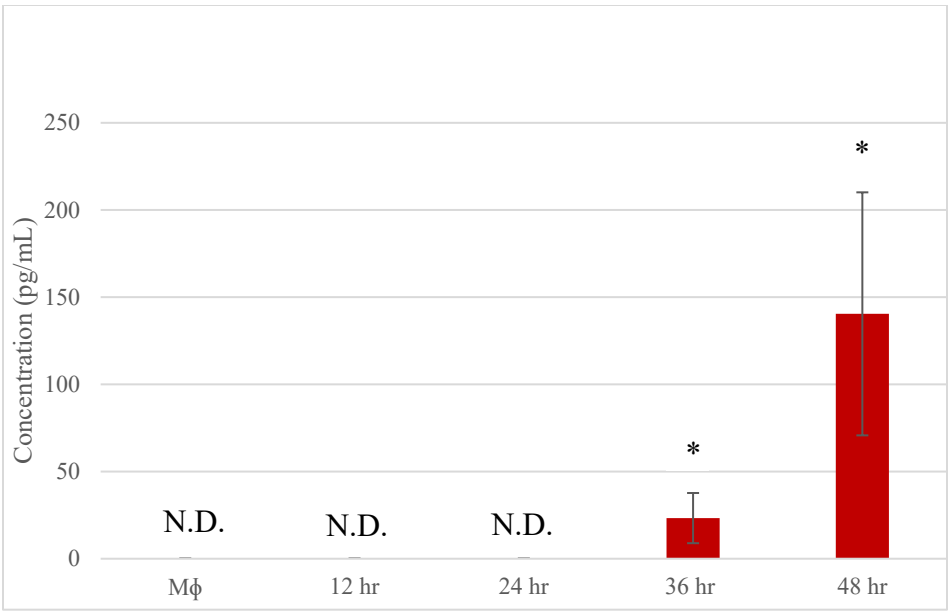


Figure 4.4 Concentration of LL-37 (pg/mL) during the co-culture of *S. epidermidis* biofilm (2.4 x 10^8 CFU/mL) with activated THP-1 (macrophage-like cells) (1.0 x 10^6) cells/mL. Aliquots 3 mL of the co-culture were extracted every 12 hours for 48 hours. After collection, the sample was prepared using SPE (C8) and analyzed using HPLC-MS/MS. Error bars denote mean ± SD, N=4 for each time point, and significantly different time points from the control where denoted by * $p<0.05$

4.3.1.3 Co-culture of *Pseudomonas aeruginosa*-biofilm and macrophage-like cells

P. aeruginosa, a Gram-negative bacterium, of high clinical significance was also analyzed using bacterial biofilm co-culture with activated THP-1 cells. However, due to the high signal-to-noise ratio (SNR), only 24 hours of co-culture was able to be measured. Like the other co-cultures, activated macrophage-like cells did not result in a detectable level of LL-37. However, when *P. aeruginosa* is introduced to the co-culture, levels of LL-37 rise to an average concentration of 1365 ± 139 pg/mL (Figure 4.5). By hour 24, levels are non-detectable again. The detection levels at 12 hours of co-culture could be due to the versatility of its virulence mechanisms [222]. This could also explain the high SNR that is seen in hours 36 and 48; At these time points, the background is so high, that LL-37 peaks cannot be extracted even in fragmentation mode, for either the quant ion

or other fragments. Other experimental protocols were attempted in an effort to measure LL-37 from *P. aeruginosa* co-cultures at time points 36 and 48 hours including smaller biofilms, protein crashing, decrease sample load on SPE, and standard addition. However, none of these approaches resulted in data that could be analyzed across 48 hours. Future work for this project will address ways to capture a more encompassing analysis of *Pseudomonas aeruginosa*, due to its clinical significance.

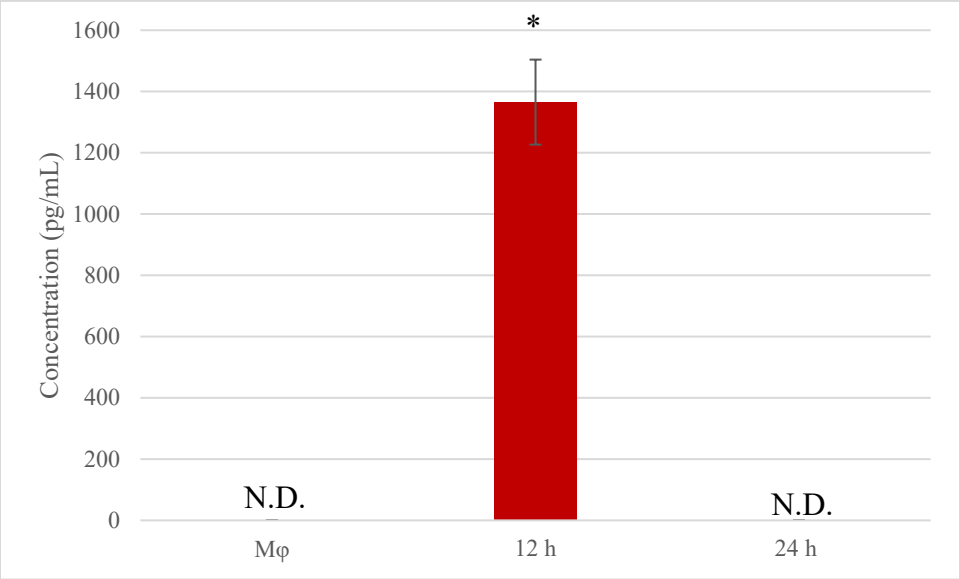


Figure 4.5 Concentration of LL-37 (pg/mL) during the co-culture of *P. aeruginosa* biofilm (3.2 x 10^8 CFU/mL) with activated THP-1 (macrophage-like cells) (1.0 x 10^6) cells/mL. Aliquots 3 mL of the co-culture were extracted every 12 hours for 24 hours. After collection, the sample was prepared using SPE (C8) and analyzed using HPLC-MS/MS. Error bars denote mean ± SD, N=4 for each time point, and significantly different time points from the control where denoted by * $p<0.05$

Table 4-1 HPLC-MS/MS areas for selected peaks during the co-culture of *P. aeruginosa* biofilm (3.2 x 108 CFU/mL) with activated THP-1 (macrophage-like cells) (1.0 x 106) cells/mL. Hours 36 and 48.

Sample ID	Retention time	Peak area
36 hours -1	-----	21,529
36 hours -2	-----	14,267
36 hours -3	-----	20,283
36 hours -4	-----	16,723
48 hours -1	-----	37,338
48 hours -2	-----	22,268
48 hours -3	-----	81,609
48 hours -4	-----	11,071

Table 4-1 shows the raw peak area data for hours 36 and 48 of the *P. aeruginosa* biofilm-macrophage-like cell co-culture established in Figure 4.5. While peak areas exist for these timepoints, no retention time could be identified; thus, it cannot be determined that the peak areas are that of LL-37, but rather a combination of LL-37 peaks, and "noise" peaks.

4.4 Conclusion

Chapter 4 focused on utilizing the sample preparation methods developed in chapter's 2 and 3 to determine levels of LL-37 during an infection state. Bacterial biofilms of *Staphylococcus aureus, Staphylococcus epidermidis*, and *Pseudomonas aeruginosa*, were co-cultured with activated THP-1 cells, which mimic macrophages. Samples underwent solid-phase extraction before HPLC-MS/MS analysis. The *S. aureus*-biofilm co-culture resulted in the "highest levels of LL-37" secretion, with an average of 7658 ± 2455 pg/mL at 12 hours. Co-culture with *S. epidermidis*-biofilms resulted in a delayed response, with levels of LL-37 being non-detectable until hour 36. This resulted in an average concentration of 23 ± 14 pg/mL. *P. aeruginosa*-biofilm

co-culture resulted in an average concentration of 1365 ± 139 pg/mL. Furthermore, this co-culture resulted in a high signal-to-noise ratio after 24 hours, making extraction of LL-37 peak fragments not possible for timepoints 36 or 48 hours.

5 Conclusion

This dissertation was aimed at developing a sample preparation method to extract and quantify LL-37 from a complex biological matrix using an *in vitro* model. As Chapter 1 denotes, healthcare-associated infections affect over 1.7 million people each year, and results in 98,000 deaths per year, and costs between 28.4 and 45 billon dollars for treatment [2]. Furthermore, these infections are a result of biofilm formation approximately 80% of the time. Among the most commonly isolated microorganisms from clinical isolates are *Staphylococcus aureus*, *Staphylococcus epidermidis*, and *Pseudomonas aeruginosa*; thus, they were the focus of this research.

Cationic antimicrobial peptides (CAMPs), also known as cathelicidins, are known to play an important role in combating infection through the elimination of pathogens, in particular, Gram-negative and Gram-positive bacteria, such as *S. aureus*, *S. epidermidis*, and *P. aeruginosa*. Cathelicidins are also known to have immunomodulatory functions such as directing chemotaxis, controlling both pro-inflammatory and anti-inflammatory states, and affecting cellular differentiation [4, 105]. This research was aimed at hCAP-18/LL-37, as it is the only known naturally occurring cathelicidin in humans. Previous studies have shown differing sample preparation methods for LL-37, each with its own advantages and disadvantages. For example, studies have shown that enzyme-linked immunosorbent assay (ELISA) can be used in the detection and quantification of LL-37 in research including ovarian cancer, lupus erythematosus, rheumatoid arthritis, ear wax, and colon epithelial cells [168-171]. Other studies have shown western blot to be an effective method in the detection of LL-37 in whole saliva, mitochondria, and type I insulin-like growth factor receptor (IGF-1R).[178-180] However, as a stand-alone

method, it is not quantitative and is very time-consuming. For this reason, this work is focused on developing a fast, reliable method for detection and quantification.

Chapter 2 focused on two different sample preparation techniques: gel electrophoresis and solid- phase extraction. Due to a known aggregation of LL-37 into both trimers and hexamers, native PAGE was explored. Several compounds, such as L-arginine, were used to determine if preventing peptide aggregation could lead to consistent results. L-arginine is commonly used in the pharmaceutical industry to prevent aggregation and extend shelf-life of medications [192]. L-Arginine (1M) proved effective in helping LL-37 move down the gel. Additionally, urea and guanidine hydrochloride (GdnHCl) were tested, to see if denaturing the peptide could lead to a more consistent result. GdnHCl (0.1-0.5 M) allowed LL-37 to travel farther down the gel than urea and was chosen as a candidate for HPLC-MS analysis. However, when samples were run on the HPLC-MS, guanidine hydrochloride supplementation did not result in a more consistent sample over-time.

Solid-phase extraction was explored as a candidate for sample preparation. In order to determine if this method was feasible, sample reconstitution and solubility first had to be investigated. Multiple solvents to increase solubility were tested, including varying concentrations of dimethyl sulfoxide (DMSO), acetonitrile (ACN), HPLC grade water, methanol (MEOH), and formic acid (FA). Solvent evaporation over time was also tested, as many of the solvents are volatile and known to evaporate quickly. From this work, it was determined that while DMSO proved viable at cleaning the HPLC column, its use as a solvent for sample stability was not viable. However, 5% FA in 60% (v/v) ACN proved a viable candidate for reconstitution and as a possible elution solvent for SPE procedures, which was further explored in Chapter 3. Finally, Chapter 2 provided a solubility range for the analyte, LL-37. It was

determined that samples of 1 ppm or less were required for analysis. This meant that all final analysis had to be done using the HPLC-MS/MS as the Bruker-Daltonics HPLC/MS could only analyze concentrations of 10 ppm or higher.

A solid-phase extraction method was developed in Chapter 3. Multiple SPE cartridges were tested with varying elution solvents to determine absolute recovery. Both the Bond-Elut PPL and SOLA WCX proved to not be viable, as recoveries were too low, and loss was detected in the load or wash. C8 cartridges were tested as an alternative to the modified C18 columns, and with the solvent established in Chapter 2, resulted in an average recovery of 82 ± 12% using 1 ppm LL-37 and 89 ± 8%, using 0.5 ppm LL-37. The limit of detection (LOD) was found to be 0.5 ppb and the limit of quantitation (LOQ) was found to be 1.6 ppb. With the established method, it was possible to move on to biological samples.

Chapter 4 encompasses the in vitro co- culture work for this dissertation. Co-cultures comprised of activated THP-1 cells and bacterial biofilms of *S. aureus, S. epidermidis*, and *P. aeruginosa* were explored to determine levels of LL-37 during an infection state. Levels of LL-37 spiked in all three bacteria, however, in *S. epidermidis*, this spike was delayed approximately 24 hours in comparison to the other bacterial strains. Furthermore, the co-culture with *P. aeruginosa* could only be run for 24 hours, as opposed to the 48 hours of the other co-cultures. The signal-to-noise ratio was too high to extract peak area. Efforts were made to counteract this, such as protein crashing, standard addition, and altering SPE load volumes. However, these attempts were unsuccessful at reducing the SNR.

Much of the future work for this project will involve determining a method to reduce the background noise seen at the end of Chapter 4. Possible solutions include the use of isotopically labelled internal standards, as they could increase reliability, and have been used in multiple

studies involving complex biological matrices [223-225]. Additionally, studies have shown that fragmenting LL-37 and varying helicities can increase the antimicrobial properties of LL-37 [158, 159]. It could be possible to use the established methods of Chapters 3 and 4 to explore alternative LL-37 therapeutic effects, with fragments of LL-37.

Furthermore, L-arginine supplementation was shown in Chapter 2 to help reduce aggregation in LL-37 standards. Preliminary data into microdialysis sampling with LL-37 standards resulted in no recovery. It was proposed that the standard was aggregating and unable to pass through the membrane due to size exclusion properties of the membrane, despite LL-37 having a low M.W. (~4500 kDa)[18]. Future work could focus on the use of L-arginine supplementation as a deaggregant to collect LL-37 both *in vitro* and *in vivo*. This work could then be used to tie in previous protocols for measuring QS molecules and various chemokines. This understanding would allow a broader immunological picture of how bacterial biofilms interact with the host during an infection state.

6 References

[1] M. Haque, M. Sartelli, J. McKimm, and M. Abu Bakar, "Health care-associated infections - an overview," (in eng), *Infection and drug resistance,* vol. 11, pp. 2321-2333, 2018, doi: 10.2147/IDR.S177247.

[2] (2014). *The Direct Medical Costs of Healthcare-Associated Infections in U.S. Hospitals and the Benefits of Prevention*.

[3] M. Jamal *et al.*, "Bacterial biofilm and associated infections," (in eng), *J Chin Med Assoc,* vol. 81, no. 1, pp. 7-11, Jan 2018, doi: 10.1016/j.jcma.2017.07.012.

[4] E. Galdiero, L. Lombardi, A. Falanga, G. Libralato, M. Guida, and R. Carotenuto, "Biofilms: Novel Strategies Based on Antimicrobial Peptides," (in eng), *Pharmaceutics,* vol. 11, no. 7, p. 322, 2019, doi: 10.3390/pharmaceutics11070322.

[5] S. L. Percival, L. Suleman, C. Vuotto, and G. Donelli, "Healthcare-associated infections, medical devices and biofilms: risk, tolerance and control," (in eng), *J Med Microbiol,* vol. 64, no. Pt 4, pp. 323-334, Apr 2015, doi: 10.1099/jmm.0.000032.

[6] Y. Cai *et al.*, "Prevalence of Healthcare-Associated Infections and Antimicrobial Use Among Adult Inpatients in Singapore Acute-Care Hospitals: Results From the First National Point Prevalence Survey," *Clinical Infectious Diseases,* vol. 64, no. suppl_2, pp. S61-S67, 2017, doi: 10.1093/cid/cix103.

[7] R. M. Donlan, "Biofilms: microbial life on surfaces," (in eng), *Emerging infectious diseases,* vol. 8, no. 9, pp. 881-890, 2002, doi: 10.3201/eid0809.020063.

[8] C. Solano, M. Echeverz, and I. Lasa, "Biofilm dispersion and quorum sensing," (in eng), *Curr Opin Microbiol,* vol. 18, pp. 96-104, Apr 2014, doi: 10.1016/j.mib.2014.02.008.

[9] G. Anderson Gregory, S. Moreau-Marquis, A. Stanton Bruce, and A. O'Toole George, "In Vitro Analysis of Tobramycin-Treated Pseudomonas aeruginosa Biofilms on Cystic Fibrosis-Derived Airway Epithelial Cells," *Infection and Immunity,* vol. 76, no. 4, pp. 1423-1433, 2008/04/01 2008, doi: 10.1128/IAI.01373-07.

[10] Y. Wu and F. W. Outten, "IscR Controls Iron-Dependent Biofilm Formation in Escherichia coli by Regulating Type I Fimbria Expression," *Journal of Bacteriology,* vol. 191, no. 4, pp. 1248-1257, 2009/02/15 2009, doi: 10.1128/JB.01086-08.

[11] W. M. Dunne, "Bacterial Adhesion: Seen Any Good Biofilms Lately?," *Clinical Microbiology Reviews,* vol. 15, no. 2, pp. 155-166, 2002/04/01 2002, doi: 10.1128/CMR.15.2.155-166.2002.

[12] E. Banin, L. Vasil Michael, and E. P. Greenberg, "Iron and Pseudomonas aeruginosa biofilm formation," *Proceedings of the National Academy of Sciences,* vol. 102, no. 31, pp. 11076-11081, 2005/08/02 2005, doi: 10.1073/pnas.0504266102.

[13] B. Carpentier and O. Cerf, "Biofilms and their consequences, with particular reference to hygiene in the food industry," *Journal of applied bacteriology,* vol. 75, no. 6, pp. 499-511, 1993.

[14] B. D. Hoyle and J. W. Costerton, "Bacterial resistance to antibiotics: the role of biofilms," (in eng), no. 0071-786X (Print).

[15] Y. H. Li and X. Tian, "Quorum sensing and bacterial social interactions in biofilms," (in eng), *Sensors (Basel),* vol. 12, no. 3, pp. 2519-38, 2012, doi: 10.3390/s120302519.

[16] M. E. Taga and B. L. Bassler, "Chemical communication among bacteria," *Proceedings of the National Academy of Sciences,* vol. 100, no. suppl 2, p. 14549, 2003, doi: 10.1073/pnas.1934514100.

[17] A. Camilli and B. L. Bassler, "Bacterial small-molecule signaling pathways," (in eng), *Science,* vol. 311, no. 5764, pp. 1113-6, Feb 24 2006, doi: 10.1126/science.1121357.
[18] W. L. Ng and B. L. Bassler, "Bacterial quorum-sensing network architectures," (in eng), *Annu Rev Genet,* vol. 43, pp. 197-222, 2009, doi: 10.1146/annurev-genet-102108-134304.
[19] S. Kırmusaoğlu, "Staphylococcal Biofilms: Pathogenicity, Mechanism and Regulation of Biofilm Formation by Quorum-Sensing System and Antibiotic Resistance Mechanisms of Biofilm-Embedded Microorganisms," 2016.
[20] Z. Qin *et al.*, "Role of autolysin-mediated DNA release in biofilm formation of Staphylococcus epidermidis," *Microbiology,* vol. 153, no. 7, pp. 2083-2092, 2007.
[21] L. Yang, K. B. Barken, M. E. Skindersoe, A. B. Christensen, M. Givskov, and T. Tolker-Nielsen, "Effects of iron on DNA release and biofilm development by Pseudomonas aeruginosa," *Microbiology,* vol. 153, no. 5, pp. 1318-1328, 2007.
[22] V. C. Thomas, L. R. Thurlow, D. Boyle, and L. E. Hancock, "Regulation of autolysis-dependent extracellular DNA release by Enterococcus faecalis extracellular proteases influences biofilm development," *Journal of bacteriology,* vol. 190, no. 16, pp. 5690-5698, 2008.
[23] P. S. Guiton *et al.*, "Contribution of autolysin and sortase A during Enterococcus faecalis DNA-dependent biofilm development," *Infection and immunity,* vol. 77, no. 9, pp. 3626-3638, 2009.
[24] S. Vilain, J. M. Pretorius, J. Theron, and V. S. Brözel, "DNA as an adhesin: Bacillus cereus requires extracellular DNA to form biofilms," *Applied and environmental microbiology,* vol. 75, no. 9, pp. 2861-2868, 2009.
[25] X. Zogaj, M. Nimtz, M. Rohde, W. Bokranz, and U. Römling, "The multicellular morphotypes of Salmonella typhimurium and Escherichia coli produce cellulose as the second component of the extracellular matrix," *Molecular microbiology,* vol. 39, no. 6, pp. 1452-1463, 2001.
[26] J. S. Pinkner *et al.*, "Rationally designed small compounds inhibit pilus biogenesis in uropathogenic bacteria," *Proceedings of the National Academy of Sciences,* vol. 103, no. 47, pp. 17897-17902, 2006.
[27] L. Cegelski *et al.*, "Small-molecule inhibitors target Escherichia coli amyloid biogenesis and biofilm formation," *Nature chemical biology,* vol. 5, no. 12, pp. 913-919, 2009.
[28] C. B. Whitchurch, T. Tolker-Nielsen, P. C. Ragas, and J. S. Mattick, "Extracellular DNA required for bacterial biofilm formation," *Science,* vol. 295, no. 5559, pp. 1487-1487, 2002.
[29] V. Palchevskiy and S. E. Finkel, "Escherichia coli competence gene homologs are essential for competitive fitness and the use of DNA as a nutrient," *Journal of bacteriology,* vol. 188, no. 11, pp. 3902-3910, 2006.
[30] J. W. Costerton, P. S. Stewart, and E. P. Greenberg, "Bacterial biofilms: a common cause of persistent infections," *science,* vol. 284, no. 5418, pp. 1318-1322, 1999.
[31] R. F. Maldonado, I. Sá-Correia, and M. A. Valvano, "Lipopolysaccharide modification in Gram-negative bacteria during chronic infection," *FEMS Microbiology Reviews,* vol. 40, no. 4, pp. 480-493, 2016, doi: 10.1093/femsre/fuw007.
[32] M. Kostakioti, M. Hadjifrangiskou, and S. J. Hultgren, "Bacterial biofilms: development, dispersal, and therapeutic strategies in the dawn of the postantibiotic era," (in eng), *Cold*

Spring Harbor perspectives in medicine, vol. 3, no. 4, pp. a010306-a010306, 2013, doi: 10.1101/cshperspect.a010306.

[33] M. Klausen *et al.*, "Biofilm formation by Pseudomonas aeruginosa wild type, flagella and type IV pili mutants," *Molecular Microbiology,* https://doi.org/10.1046/j.1365-2958.2003.03525.x vol. 48, no. 6, pp. 1511-1524, 2003/06/01 2003, doi: https://doi.org/10.1046/j.1365-2958.2003.03525.x.

[34] I. Vallet, W. Olson John, S. Lory, A. Lazdunski, and A. Filloux, "The chaperone/usher pathways of Pseudomonas aeruginosa: Identification of fimbrial gene clusters (cup) and their involvement in biofilm formation," *Proceedings of the National Academy of Sciences,* vol. 98, no. 12, pp. 6911-6916, 2001/06/05 2001, doi: 10.1073/pnas.111551898.

[35] J. Klebensberger, A. Birkenmaier, R. Geffers, S. Kjelleberg, and B. Philipp, "SiaA and SiaD are essential for inducing autoaggregation as a specific response to detergent stress in Pseudomonas aeruginosa," *Environmental Microbiology,* https://doi.org/10.1111/j.1462-2920.2009.02012.x vol. 11, no. 12, pp. 3073-3086, 2009/12/01 2009, doi: https://doi.org/10.1111/j.1462-2920.2009.02012.x.

[36] G. A. O'Toole and R. Kolter, "Flagellar and twitching motility are necessary for Pseudomonas aeruginosa biofilm development," *Molecular Microbiology,* https://doi.org/10.1046/j.1365-2958.1998.01062.x vol. 30, no. 2, pp. 295-304, 1998/10/01 1998, doi: https://doi.org/10.1046/j.1365-2958.1998.01062.x.

[37] M. Fletcher, "Bacterial attachment in aquatic environments: a diversity of surfaces and adhesion strategies," *Bacterial adhesion: molecular and ecological diver-sity.,* pp. 1-24, 1993.

[38] I. A. Myles and S. K. Datta, "Staphylococcus aureus: an introduction," (in eng), *Seminars in immunopathology,* vol. 34, no. 2, pp. 181-184, 2012, doi: 10.1007/s00281-011-0301-9.

[39] E. P. Skaar, M. Humayun, T. Bae, K. L. DeBord, and O. Schneewind, "Iron-source preference of Staphylococcus aureus infections," *Science,* vol. 305, no. 5690, pp. 1626-1628, 2004.

[40] J. Meiwes, H. P. Fiedler, H. Haag, H. Zähner, S. Konetschny-Rapp, and G. Jung, "Isolation and characterization of staphyloferrin A, a compound with siderophore activity from Staphylococcus hyicus DSM 20459," *FEMS microbiology letters,* vol. 67, no. 1-2, pp. 201-205, 1990.

[41] J. Bubeck Wardenburg, T. Bae, M. Otto, F. R. DeLeo, and O. Schneewind, "Poring over pores: α-hemolysin and Panton-Valentine leukocidin in Staphylococcus aureus pneumonia," *Nature medicine,* vol. 13, no. 12, pp. 1405-1406, 2007.

[42] A. D. Kennedy *et al.*, "Targeting of alpha-hemolysin by active or passive immunization decreases severity of USA300 skin infection in a mouse model," *The Journal of infectious diseases,* vol. 202, no. 7, pp. 1050-1058, 2010.

[43] F. C. Beasley *et al.*, "Characterization of staphyloferrin A biosynthetic and transport mutants in Staphylococcus aureus," *Molecular microbiology,* vol. 72, no. 4, pp. 947-963, 2009.

[44] M. J. Horsburgh, E. Ingham, and S. J. Foster, "In Staphylococcus aureus, Fur is an interactive regulator with PerR, contributes to virulence, and is necessary for oxidative stress resistance through positive regulation of catalase and iron homeostasis," *Journal of bacteriology,* vol. 183, no. 2, pp. 468-475, 2001.

[45] P. M. Alves, E. Al-Badi, C. Withycombe, P. M. Jones, K. J. Purdy, and S. E. Maddocks, "Interaction between Staphylococcus aureus and Pseudomonas aeruginosa is beneficial for colonisation and pathogenicity in a mixed biofilm. LID - 10.1093/femspd/fty003 [doi]," (in eng), no. 2049-632X (Electronic).
[46] V. G. Preda and O. Săndulescu, "Communication is the key: biofilms, quorum sensing, formation and prevention," (in eng), *Discoveries (Craiova, Romania),* vol. 7, no. 3, pp. e100-e100, 2019, doi: 10.15190/d.2019.13.
[47] R. Daniels, J. Vanderleyden, and J. Michiels, "Quorum sensing and swarming migration in bacteria," *FEMS Microbiology Reviews,* vol. 28, no. 3, pp. 261-289, 2004, doi: 10.1016/j.femsre.2003.09.004.
[48] S. Mukherjee and B. L. Bassler, "Bacterial quorum sensing in complex and dynamically changing environments," (in eng), *Nat Rev Microbiol,* vol. 17, no. 6, pp. 371-382, Jun 2019, doi: 10.1038/s41579-019-0186-5.
[49] M. B. Miller and B. L. Bassler, "Quorum sensing in bacteria," (in eng), *Annu Rev Microbiol,* vol. 55, pp. 165-99, 2001, doi: 10.1146/annurev.micro.55.1.165.
[50] S. T. Rutherford and B. L. Bassler, "Bacterial quorum sensing: its role in virulence and possibilities for its control," (in eng), *Cold Spring Harb Perspect Med,* vol. 2, no. 11, Nov 1 2012, doi: 10.1101/cshperspect.a012427.
[51] J. Lin, J. Cheng, Y. Wang, and X. Shen, "The Pseudomonas Quinolone Signal (PQS): Not Just for Quorum Sensing Anymore," (in eng), *Frontiers in cellular and infection microbiology,* vol. 8, pp. 230-230, 2018, doi: 10.3389/fcimb.2018.00230.
[52] J. Lee and L. Zhang, "The hierarchy quorum sensing network in Pseudomonas aeruginosa," (in eng), *Protein & cell,* vol. 6, no. 1, pp. 26-41, 2015, doi: 10.1007/s13238-014-0100-x.
[53] R. Dandela, D. Mantin, B. F. Cravatt, J. Rayo, and Michael M. Meijler, "Proteome-wide mapping of PQS-interacting proteins in Pseudomonas aeruginosa," *Chemical Science,* 10.1039/C7SC04287F vol. 9, no. 8, pp. 2290-2294, 2018, doi: 10.1039/C7SC04287F.
[54] Y. R. Baker *et al.*, "Identification of new quorum sensing autoinducer binding partners in Pseudomonas aeruginosa using photoaffinity probes," *Chemical Science,* 10.1039/C7SC01270E vol. 8, no. 11, pp. 7403-7411, 2017, doi: 10.1039/C7SC01270E.
[55] J. T. Hodgkinson, J. Gross, Y. R. Baker, D. R. Spring, and M. Welch, "A new Pseudomonas quinolone signal (PQS) binding partner: MexG," *Chemical Science,* 10.1039/C5SC04197J vol. 7, no. 4, pp. 2553-2562, 2016, doi: 10.1039/C5SC04197J.
[56] M. Davinic, N. L. Carty, J. A. Colmer-Hamood, M. San Francisco, and A. N. Hamood, "Role of Vfr in regulating exotoxin A production by Pseudomonas aeruginosa," (in eng), *Microbiology (Reading, England),* vol. 155, no. Pt 7, pp. 2265-2273, 2009, doi: 10.1099/mic.0.028373-0.
[57] L. E. P. Dietrich, A. Price-Whelan, A. Petersen, M. Whiteley, and D. K. Newman, "The phenazine pyocyanin is a terminal signalling factor in the quorum sensing network of Pseudomonas aeruginosa," *Molecular Microbiology,* https://doi.org/10.1111/j.1365-2958.2006.05306.x vol. 61, no. 5, pp. 1308-1321, 2006/09/01 2006, doi: https://doi.org/10.1111/j.1365-2958.2006.05306.x.
[58] E. Kay *et al.*, "Two GacA-dependent small RNAs modulate the quorum-sensing response in Pseudomonas aeruginosa," (in eng), no. 0021-9193 (Print).

[59] W. Lau Gee, H. Ran, F. Kong, J. Hassett Daniel, and D. Mavrodi, "Pseudomonas aeruginosa Pyocyanin Is Critical for Lung Infection in Mice," *Infection and Immunity,* vol. 72, no. 7, pp. 4275-4278, 2004/07/01 2004, doi: 10.1128/IAI.72.7.4275-4278.2004.
[60] E. R. Morris *et al.*, "Structural rearrangement in an RsmA/CsrA ortholog of Pseudomonas aeruginosa creates a dimeric RNA-binding protein, RsmN," (in eng), *Structure (London, England : 1993),* vol. 21, no. 9, pp. 1659-1671, 2013, doi: 10.1016/j.str.2013.07.007.
[61] H. Sakhtah *et al.*, "The Pseudomonas aeruginosa efflux pump MexGHI-OpmD transports a natural phenazine that controls gene expression and biofilm development," (in eng), no. 1091-6490 (Electronic).
[62] E. K. Shiner *et al.*, "Pseudomonas aeruginosa autoinducer modulates host cell responses through calcium signalling," *Cellular microbiology,* vol. 8, no. 10, pp. 1601-1610, 2006.
[63] V. E. Wagner, J. G. Frelinger, R. K. Barth, and B. H. Iglewski, "Quorum sensing: dynamic response of Pseudomonas aeruginosa to external signals," *Trends in microbiology,* vol. 14, no. 2, pp. 55-58, 2006.
[64] S. E. Turvey and D. H. Broide, "Innate immunity," *Journal of Allergy and Clinical Immunology,* vol. 125, no. 2, Supplement 2, pp. S24-S32, 2010/02/01/ 2010, doi: https://doi.org/10.1016/j.jaci.2009.07.016.
[65] F. A. Bonilla and H. C. Oettgen, "Adaptive immunity," *Journal of Allergy and Clinical Immunology,* vol. 125, no. 2, Supplement 2, pp. S33-S40, 2010/02/01/ 2010, doi: https://doi.org/10.1016/j.jaci.2009.09.017.
[66] J. S. Marshall, R. Warrington, W. Watson, and H. L. Kim, "An introduction to immunology and immunopathology," *Allergy, Asthma & Clinical Immunology,* vol. 14, no. 2, p. 49, 2018/09/12 2018, doi: 10.1186/s13223-018-0278-1.
[67] P. Krzyszczyk, R. Schloss, A. Palmer, and F. Berthiaume, "The Role of Macrophages in Acute and Chronic Wound Healing and Interventions to Promote Pro-wound Healing Phenotypes," *Frontiers in Physiology,* Review vol. 9, 2018.
[68] D. Hirayama, T. Iida, and H. Nakase, "The Phagocytic Function of Macrophage-Enforcing Innate Immunity and Tissue Homeostasis," (in eng), *International journal of molecular sciences,* vol. 19, no. 1, p. 92, 2017, doi: 10.3390/ijms19010092.
[69] M. Orecchioni, Y. Ghosheh, A. B. Pramod, and K. Ley, "Macrophage Polarization: Different Gene Signatures in M1(LPS+) vs. Classically and M2(LPS–) vs. Alternatively Activated Macrophages," *Frontiers in Immunology,* Review vol. 10, 2019.
[70] D. K. Fogg *et al.*, "A clonogenic bone marrow progenitor specific for macrophages and dendritic cells," in *Science*, vol. 311, no. 5757). United States, 2006, pp. 83-7.
[71] S. Gordon and P. R. Taylor, "Monocyte and macrophage heterogeneity," *Nature Reviews Immunology,* vol. 5, no. 12, pp. 953-964, 2005/12/01 2005, doi: 10.1038/nri1733.
[72] D. A. Hume, I. L. Ross, S. R. Himes, R. T. Sasmono, C. A. Wells, and T. Ravasi, "The mononuclear phagocyte system revisited," (in eng), *J Leukoc Biol,* vol. 72, no. 4, pp. 621-7, Oct 2002.
[73] F. O. Martinez, A. Sica, A. Mantovani, and M. Locati, "Macrophage activation and polarization," (in eng), *Front Biosci,* vol. 13, pp. 453-61, Jan 1 2008, doi: 10.2741/2692.
[74] C. Atri, F. Z. Guerfali, and D. Laouini, "Role of Human Macrophage Polarization in Inflammation during Infectious Diseases," (in eng), *International journal of molecular sciences,* vol. 19, no. 6, p. 1801, 2018, doi: 10.3390/ijms19061801.

[75] M. E. Martinez-Sanchez, L. Huerta, E. R. Alvarez-Buylla, and C. Villarreal Luján, "Role of Cytokine Combinations on CD4+ T Cell Differentiation, Partial Polarization, and Plasticity: Continuous Network Modeling Approach," (in eng), no. 1664-042X (Print).
[76] D. Y. Vogel *et al.*, "Human macrophage polarization in vitro: maturation and activation methods compared," in *Immunobiology*, vol. 219, no. 9). Netherlands: © 2014 Elsevier GmbH, 2014, pp. 695-703.
[77] S. Iqbal and A. Kumar, "Characterization of In vitro Generated Human Polarized Macrophages," *Journal of Clinical & Cellular Immunology,* vol. 06, 01/01 2015, doi: 10.4172/2155-9899.1000380.
[78] D. Duluc *et al.*, "Tumor-associated leukemia inhibitory factor and IL-6 skew monocyte differentiation into tumor-associated macrophage-like cells," *Blood,* vol. 110, no. 13, pp. 4319-4330, 2007, doi: 10.1182/blood-2007-02-072587.
[79] J.-F. Viallard *et al.*, "CD8+HLA-DR+ T lymphocytes are increased in common variable immunodeficiency patients with impaired memory B-cell differentiation," *Clinical Immunology,* vol. 119, no. 1, pp. 51-58, 2006/04/01/ 2006, doi: https://doi.org/10.1016/j.clim.2005.11.011.
[80] C. Baecher-Allan, D. A. Wolf E Fau - Hafler, and D. A. Hafler, "MHC class II expression identifies functionally distinct human regulatory T cells," (in eng), no. 0022-1767 (Print).
[81] D. P. Saraiva, A. Jacinto, P. Borralho, S. Braga, and M. G. Cabral, "HLA-DR in Cytotoxic T Lymphocytes Predicts Breast Cancer Patients' Response to Neoadjuvant Chemotherapy," (in eng), no. 1664-3224 (Electronic).
[82] F. O. Martinez, A. Sica, A. Mantovani, and M. Locati, "Macrophage activation and polarization," *Front. Biosci.,* 10.2741/2692 vol. 13, pp. 453-461, // 2008, doi: 10.2741/2692.
[83] A. Mantovani, M. Sozzani S Fau - Locati, P. Locati M Fau - Allavena, A. Allavena P Fau - Sica, and A. Sica, "Macrophage polarization: tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes," (in eng), no. 1471-4906 (Print).
[84] T. Pannellini *et al.*, "The expression of LEC/CCL16, a powerful inflammatory chemokine, is upregulated in ulcerative colitis," (in eng), no. 0394-6320 (Print).
[85] H. Bosshart and M. Heinzelmann, "Endotoxin-neutralizing effects of histidine-rich peptides," *FEBS Letters,* vol. 553, no. 1, pp. 135-140, 2003/10/09/ 2003, doi: https://doi.org/10.1016/S0014-5793(03)00997-9.
[86] H. Bosshart and M. Heinzelmann, "THP-1 cells as a model for human monocytes," (in eng), *Annals of translational medicine,* vol. 4, no. 21, pp. 438-438, 2016, doi: 10.21037/atm.2016.08.53.
[87] S. Tsuchiya, M. Yamabe, Y. Yamaguchi, Y. Kobayashi, T. Konno, and K. Tada, "Establishment and characterization of a human acute monocytic leukemia cell line (THP-1)," *International Journal of Cancer,* https://doi.org/10.1002/ijc.2910260208 vol. 26, no. 2, pp. 171-176, 1980/08/15 1980, doi: https://doi.org/10.1002/ijc.2910260208.
[88] Z.-D. Hu *et al.*, "Gene expression profile of THP-1 cells treated with heat-killed Candida albicans," (in eng), *Annals of translational medicine,* vol. 4, no. 9, pp. 170-170, 2016, doi: 10.21037/atm.2016.05.03.
[89] J. Auwerx, "The human leukemia cell line, THP-1: a multifacetted model for the study of monocyte-macrophage differentiation," (in eng), *Experientia,* vol. 47, no. 1, pp. 22-31, Jan 15 1991, doi: 10.1007/bf02041244.

[90] H. Schwende, E. Fitzke, P. Ambs, and P. Dieter, "Differences in the state of differentiation of THP-1 cells induced by phorbol ester and 1,25-dihydroxyvitamin D3," (in eng), *J Leukoc Biol,* vol. 59, no. 4, pp. 555-61, Apr 1996.
[91] M. Daigneault, H. M. Preston Ja Fau - Marriott, M. K. B. Marriott Hm Fau - Whyte, D. H. Whyte Mk Fau - Dockrell, and D. H. Dockrell, "The identification of markers of macrophage differentiation in PMA-stimulated THP-1 cells and monocyte-derived macrophages," (in eng), no. 1932-6203 (Electronic).
[92] I. Olsson, U. Gullberg, I. Ivhed, and K. Nilsson, "Induction of Differentiation of the Human Histiocytic Lymphoma Cell Line U-937 by 1α,25-Dihydroxycholecalciferol1," *Cancer Research,* vol. 43, no. 12_Part_1, pp. 5862-5867, 1983.
[93] S.-i. Murao, M. A. Gemmell, M. F. Callaham, N. L. Anderson, and E. Huberman, "Control of Macrophage Cell Differentiation in Human Promyelocytic HL-60 Leukemia Cells by 1,25-Dihydroxyvitamin D3 and Phorbol-12-myristate-13-acetate1," *Cancer Research,* vol. 43, no. 10, pp. 4989-4996, 1983.
[94] M. E. Lund, J. To, B. A. O'Brien, and S. Donnelly, "The choice of phorbol 12-myristate 13-acetate differentiation protocol influences the response of THP-1 macrophages to a pro-inflammatory stimulus," (in eng), *J Immunol Methods,* vol. 430, pp. 64-70, Mar 2016, doi: 10.1016/j.jim.2016.01.012.
[95] M. B. Maeß, B. Wittig, A. Cignarella, and S. Lorkowski, "Reduced PMA enhances the responsiveness of transfected THP-1 macrophages to polarizing stimuli," (in eng), *J Immunol Methods,* vol. 402, no. 1-2, pp. 76-81, Jan 15 2014, doi: 10.1016/j.jim.2013.11.006.
[96] P. Crauwels *et al.*, (in eng).
[97] R. A. Dorschner, K. H. Lin, M. Murakami, and R. L. Gallo, "Neonatal Skin in Mice and Humans Expresses Increased Levels of Antimicrobial Peptides: Innate Immunity During Development of the Adaptive Response," *Pediatric Research,* vol. 53, no. 4, pp. 566-572, 2003/04/01 2003, doi: 10.1203/01.PDR.0000057205.64451.B7.
[98] R. A. Dorschner, J. Lopez-Garcia B Fau - Massie, C. Massie J Fau - Kim, R. L. Kim C Fau - Gallo, and R. L. Gallo, "Innate immune defense of the nail unit by antimicrobial peptides," (in eng), no. 0190-9622 (Print).
[99] R. E. W. Hancock and H.-G. Sahl, "Antimicrobial and host-defense peptides as new anti-infective therapeutic strategies," *Nature Biotechnology,* vol. 24, no. 12, pp. 1551-1557, 2006/12/01 2006, doi: 10.1038/nbt1267.
[100] T. Hirsch, H.-U. Jacobsen F Fau - Steinau, L. Steinau Hu Fau - Steinstraesser, and L. Steinstraesser, "Host defense peptides and the new line of defence against multiresistant infections," (in eng), no. 0929-8665 (Print).
[101] R. I. Lehrer and T. Ganz, "Antimicrobial peptides in mammalian and insect host defence," *Current Opinion in Immunology,* vol. 11, no. 1, pp. 23-27, 1999/02/01/ 1999, doi: https://doi.org/10.1016/S0952-7915(99)80005-3.
[102] I. A. Edwards, A. G. Elliott, A. M. Kavanagh, J. Zuegg, M. A. T. Blaskovich, and M. A. Cooper, "Contribution of Amphipathicity and Hydrophobicity to the Antimicrobial Activity and Cytotoxicity of β-Hairpin Peptides," (in eng), *ACS infectious diseases,* vol. 2, no. 6, pp. 442-450, 2016, doi: 10.1021/acsinfecdis.6b00045.
[103] R. M. van Harten, E. van Woudenbergh, A. van Dijk, and H. P. Haagsman, "Cathelicidins: Immunomodulatory Antimicrobials," (in eng), *Vaccines,* vol. 6, no. 3, p. 63, 2018, doi: 10.3390/vaccines6030063.

[104] A. Hollmann *et al.*, "Role of amphipathicity and hydrophobicity in the balance between hemolysis and peptide-membrane interactions of three related antimicrobial peptides," (in eng), no. 1873-4367 (Electronic).
[105] J. Overhage, A. Campisano, M. Bains, E. C. Torfs, B. H. Rehm, and R. E. Hancock, "Human host defense peptide LL-37 prevents bacterial biofilm formation," (in eng), *Infect Immun,* vol. 76, no. 9, pp. 4176-82, Sep 2008, doi: 10.1128/iai.00318-08.
[106] E. M. Kościuczuk *et al.*, "Cathelicidins: family of antimicrobial peptides. A review," *Molecular Biology Reports,* vol. 39, no. 12, pp. 10957-10970, 2012/12/01 2012, doi: 10.1007/s11033-012-1997-x.
[107] J. Agier, M. Efenberger, and E. Brzezińska-Błaszczyk, "Cathelicidin impact on inflammatory cells," *Central-European journal of immunology,* vol. 40, no. 2, p. 225, 2015.
[108] M. Zanetti, "The role of cathelicidins in the innate host defenses of mammals," *Current issues in molecular biology,* vol. 7, no. 2, pp. 179-196, 2005.
[109] J. M. Ageitos, A. Sánchez-Pérez, P. Calo-Mata, and T. G. Villa, "Antimicrobial peptides (AMPs): Ancient compounds that represent novel weapons in the fight against bacteria," *Biochemical pharmacology,* vol. 133, pp. 117-138, 2017.
[110] R. E. W. Hancock, E. F. Haney, and E. E. Gill, "The immunology of host defence peptides: beyond antimicrobial activity," *Nature Reviews Immunology,* vol. 16, no. 5, pp. 321-334, 2016.
[111] A. L. Hilchie, K. Wuerth, and R. E. W. Hancock, "Immune modulation by multifaceted cationic host defense (antimicrobial) peptides," *Nature chemical biology,* vol. 9, no. 12, pp. 761-768, 2013.
[112] S. C. Mansour, O. M. Pena, and R. E. W. Hancock, "Host defense peptides: front-line immunomodulators," *Trends in immunology,* vol. 35, no. 9, pp. 443-450, 2014.
[113] R. M. Epand and H. J. Vogel, "Diversity of antimicrobial peptides and their mechanisms of action," *Biochimica et Biophysica Acta (BBA) - Biomembranes,* vol. 1462, no. 1, pp. 11-28, 1999/12/15/ 1999, doi: https://doi.org/10.1016/S0005-2736(99)00198-4.
[114] R. Bals, M. Wang X Fau - Zasloff, J. M. Zasloff M Fau - Wilson, and J. M. Wilson, "The peptide antibiotic LL-37/hCAP-18 is expressed in epithelia of the human lung where it has broad antimicrobial activity at the airway surface," (in eng), no. 0027-8424 (Print).
[115] G. H. Gudmundsson, J. Agerberth B Fau - Odeberg, T. Odeberg J Fau - Bergman, B. Bergman T Fau - Olsson, R. Olsson B Fau - Salcedo, and R. Salcedo, "The human gene FALL39 and processing of the cathelin precursor to the antibacterial peptide LL-37 in granulocytes," (in eng), no. 0014-2956 (Print).
[116] J. M. Kahlenberg and M. J. Kaplan, "Little peptide, big effects: the role of LL-37 in inflammation and autoimmune disease," (in eng), no. 1550-6606 (Electronic).
[117] A. M. van der Does *et al.*, "LL-37 directs macrophage differentiation toward macrophages with a proinflammatory signature," (in eng), no. 1550-6606 (Electronic).
[118] P. Bergman, L. Walter-Jallow, K. Broliden, B. Agerberth, and J. Söderlund, "The antimicrobial peptide LL-37 inhibits HIV-1 replication," (in eng), *Curr HIV Res,* vol. 5, no. 4, pp. 410-5, Jul 2007, doi: 10.2174/157016207781023947.
[119] Z. Hu *et al.*, "Antimicrobial cathelicidin peptide LL-37 inhibits the LPS/ATP-induced pyroptosis of macrophages by dual mechanism," (in eng), *PloS one,* vol. 9, no. 1, pp. e85765-e85765, 2014, doi: 10.1371/journal.pone.0085765.

[120] V. K. Lishko, B. Moreno, N. P. Podolnikova, and T. P. Ugarova, "Identification of Human Cathelicidin Peptide LL-37 as a Ligand for Macrophage Integrin α(M)β(2) (Mac-1, CD11b/CD18) that Promotes Phagocytosis by Opsonizing Bacteria," (in eng), *Res Rep Biochem,* vol. 2016, no. 6, pp. 39-55, Jul 7 2016, doi: 10.2147/rrbc.s107070.
[121] S. Paul and G. Lal, "The Molecular Mechanism of Natural Killer Cells Function and Its Importance in Cancer Immunotherapy," *Frontiers in Immunology,* Review vol. 8, 2017.
[122] U. H. Dürr, U. S. Sudheendra, and A. Ramamoorthy, "LL-37, the only human member of the cathelicidin family of antimicrobial peptides," (in eng), *Biochim Biophys Acta,* vol. 1758, no. 9, pp. 1408-25, Sep 2006, doi: 10.1016/j.bbamem.2006.03.030.
[123] B. Agerberth *et al.*, "The human antimicrobial and chemotactic peptides LL-37 and alpha-defensins are expressed by specific lymphocyte and monocyte populations," (in eng), no. 0006-4971 (Print).
[124] B. Agerberth *et al.*, "Antibacterial components in bronchoalveolar lavage fluid from healthy individuals and sarcoidosis patients," (in eng), no. 1073-449X (Print).
[125] B. Agerberth, H. Gunne, J. Odeberg, P. Kogner, H. G. Boman, and G. H. Gudmundsson, "FALL-39, a putative human peptide antibiotic, is cysteine-free and expressed in bone marrow and testis," (in eng), *Proceedings of the National Academy of Sciences of the United States of America,* vol. 92, no. 1, pp. 195-199, 1995, doi: 10.1073/pnas.92.1.195.
[126] H. T. Akinbi, A. K. Narendran V Fau - Pass, P. Pass Ak Fau - Markart, S. B. Markart P Fau - Hoath, and S. B. Hoath, "Host defense proteins in vernix caseosa and amniotic fluid," (in eng), no. 0002-9378 (Print).
[127] E. Andersson, O. E. Sørensen, B. Frohm, N. Borregaard, A. Egesten, and J. Malm, "Isolation of human cationic antimicrobial protein-18 from seminal plasma and its association with prostasomes," *Human Reproduction,* vol. 17, no. 10, pp. 2529-2534, 2002, doi: 10.1093/humrep/17.10.2529.
[128] S. A. Armogida, A. L. Yannaras Nm Fau - Melton, M. D. Melton Al Fau - Srivastava, and M. D. Srivastava, "Identification and quantification of innate immune system mediators in human breast milk," (in eng), no. 1088-5412 (Print).
[129] P. H. Chen and S. Y. Fang, "The expression of human antimicrobial peptide LL-37 in the human nasal mucosa," (in eng), no. 1050-6586 (Print).
[130] B. A. Dale *et al.*, "Localized antimicrobial peptide expression in human gingiva," *Journal of periodontal research,* vol. 36, no. 5, pp. 285-294, 2001.
[131] M. Frohm Nilsson, B. Sandstedt, O. Sørensen, G. Weber, N. Borregaard, and M. Ståhle-Bäckdahl, "The human cationic antimicrobial protein (hCAP18), a peptide antibiotic, is widely expressed in human squamous epithelia and colocalizes with interleukin-6," (in eng), *Infection and immunity,* vol. 67, no. 5, pp. 2561-2566, 1999, doi: 10.1128/IAI.67.5.2561-2566.1999.
[132] M. Frohm *et al.*, "Biochemical and antibacterial analysis of human wound and blister fluid," (in eng), no. 0014-2956 (Print).
[133] Y. J. Gordon, L. C. Huang, E. G. Romanowski, K. A. Yates, R. J. Proske, and A. M. McDermott, "Human cathelicidin (LL-37), a multifunctional peptide, is expressed by ocular surface epithelia and has potent antibacterial and antiviral activity," (in eng), *Current eye research,* vol. 30, no. 5, pp. 385-394, 2005, doi: 10.1080/02713680590934111.
[134] K. Hase, J. D. Eckmann L Fau - Leopard, N. Leopard Jd Fau - Varki, M. F. Varki N Fau - Kagnoff, and M. F. Kagnoff, "Cell differentiation is a key determinant of cathelicidin LL-

37/human cationic antimicrobial protein 18 expression by human colon epithelium," (in eng), no. 0019-9567 (Print).
[135] J. Malm *et al.*, "The human cationic antimicrobial protein (hCAP-18) is expressed in the epithelium of human epididymis, is present in seminal plasma at high concentrations, and is attached to spermatozoa," (in eng), no. 0019-9567 (Print).
[136] G. Marchini *et al.*, "The newborn infant is protected by an innate antimicrobial barrier: peptide antibiotics are present in the skin and vernix caseosa," (in eng), no. 0007-0963 (Print).
[137] M. Murakami, T. Ohtake, R. A. Dorschner, R. L. Gallo, B. Schittek, and C. Garbe, "Cathelicidin Anti-Microbial Peptide Expression in Sweat, an Innate Defense System for the Skin," *Journal of Investigative Dermatology,* vol. 119, no. 5, pp. 1090-1095, 2002/11/01/ 2002, doi: https://doi.org/10.1046/j.1523-1747.2002.19507.x.
[138] M. Murakami, R. A. Ohtake T Fau - Dorschner, R. L. Dorschner Ra Fau - Gallo, and R. L. Gallo, "Cathelicidin antimicrobial peptides are expressed in salivary glands and saliva," (in eng), no. 0022-0345 (Print).
[139] M. Murakami, L. J. Dorschner Ra Fau - Stern, K. H. Stern Lj Fau - Lin, R. L. Lin Kh Fau - Gallo, and R. L. Gallo, "Expression and secretion of cathelicidin antimicrobial peptides in murine mammary glands and human milk," (in eng), no. 0031-3998 (Print).
[140] F. Paulsen *et al.*, "Antimicrobial peptides are expressed and produced in healthy and inflamed human synovial membranes," (in eng), no. 0022-3417 (Print).
[141] J. Schauber *et al.*, "Expression of the cathelicidin LL-37 is modulated by short chain fatty acids in colonocytes: relevance of signalling pathways," (in eng), *Gut,* vol. 52, no. 5, pp. 735-741, 2003, doi: 10.1136/gut.52.5.735.
[142] T. D. Starner, B. Agerberth, G. H. Gudmundsson, and P. B. McCray, "Expression and Activity of β-Defensins and LL-37 in the Developing Human Lung," *The Journal of Immunology,* vol. 174, no. 3, p. 1608, 2005, doi: 10.4049/jimmunol.174.3.1608.
[143] M. Tollin, T. Bergman P Fau - Svenberg, H. Svenberg T Fau - Jörnvall, G. H. Jörnvall H Fau - Gudmundsson, B. Gudmundsson Gh Fau - Agerberth, and B. Agerberth, "Antimicrobial peptides in the first line defence of human colon mucosa," (in eng), no. 0196-9781 (Print).
[144] M. Tollin *et al.*, "Vernix caseosa as a multi-component defence system based on polypeptides, lipids and their interactions," (in eng), *Cellular and molecular life sciences : CMLS,* vol. 62, no. 19-20, pp. 2390-2399, 2005, doi: 10.1007/s00018-005-5260-7.
[145] H. Yoshio *et al.*, "Antimicrobial polypeptides of human vernix caseosa and amniotic fluid: implications for newborn innate defense," (in eng), no. 0031-3998 (Print).
[146] G. Wang, "Structures of human host defense cathelicidin LL-37 and its smallest antimicrobial peptide KR-12 in lipid micelles," (in eng), *J Biol Chem,* vol. 283, no. 47, pp. 32637-43, Nov 21 2008, doi: 10.1074/jbc.M805533200.
[147] J.-S. Woo, J. Y. Jeong, Y. J. Hwang, S. W. Chae, S. J. Hwang, and H.-M. Lee, "Expression of Cathelicidin in Human Salivary Glands," *Archives of Otolaryngology–Head & Neck Surgery,* vol. 129, no. 2, pp. 211-214, 2003, doi: 10.1001/archotol.129.2.211.
[148] K. Kuroda, K. Okumura, H. Isogai, and E. Isogai, "The Human Cathelicidin Antimicrobial Peptide LL-37 and Mimics are Potential Anticancer Drugs," *Frontiers in Oncology,* Review vol. 5, 2015.

[149] A. Diaz Perez, "Developing a Microdialysis Sampling-based Biofilm/Macrophage Co-Culture Model," 2021.
[150] M. Zasloff, "Antimicrobial peptides of multicellular organisms," (in eng), *Nature,* vol. 415, no. 6870, pp. 389-95, Jan 24 2002, doi: 10.1038/415389a.
[151] J. B. McPhee and R. E. Hancock, "Function and therapeutic potential of host defence peptides," (in eng), *J Pept Sci,* vol. 11, no. 11, pp. 677-87, Nov 2005, doi: 10.1002/psc.704.
[152] A. J. Duplantier and M. L. van Hoek, "The Human Cathelicidin Antimicrobial Peptide LL-37 as a Potential Treatment for Polymicrobial Infected Wounds," (in eng), no. 1664-3224 (Print).
[153] M. Yoshioka *et al.*, "Human cathelicidin CAP18/LL-37 changes mast cell function toward innate immunity," (in eng), no. 0918-6158 (Print).
[154] I. Nagaoka, H. Tamura, and M. Hirata, "An Antimicrobial Cathelicidin Peptide, Human CAP18/LL-37, Suppresses Neutrophil Apoptosis via the Activation of Formyl-Peptide Receptor-Like 1 and P2X₇," *The Journal of Immunology,* vol. 176, no. 5, p. 3044, 2006, doi: 10.4049/jimmunol.176.5.3044.
[155] U. Department of Pathology and Microbiology, "The Antimicrobial Peptide Database," ed, 2003-2020.
[156] G. Wang, B. Mishra, R. F. Epand, and R. M. Epand, "High-quality 3D structures shine light on antibacterial, anti-biofilm and antiviral activities of human cathelicidin LL-37 and its fragments," *Biochimica et Biophysica Acta (BBA) - Biomembranes,* vol. 1838, no. 9, pp. 2160-2172, 2014/09/01/ 2014, doi: https://doi.org/10.1016/j.bbamem.2014.01.016.
[157] J. B. Cowland, N. Johnsen Ah Fau - Borregaard, and N. Borregaard, "hCAP-18, a cathelin/pro-bactenecin-like protein of human neutrophil specific granules," (in eng), no. 0014-5793 (Print).
[158] M. F. Burton and P. G. Steel, "The chemistry and biology of LL-37," *Natural Product Reports,* 10.1039/B912533G vol. 26, no. 12, pp. 1572-1584, 2009, doi: 10.1039/B912533G.
[159] M. H. Braff *et al.*, "Structure-Function Relationships among Human Cathelicidin Peptides: Dissociation of Antimicrobial Properties from Host Immunostimulatory Activities," *The Journal of Immunology,* vol. 174, no. 7, p. 4271, 2005, doi: 10.4049/jimmunol.174.7.4271.
[160] O. E. Sørensen *et al.*, "Human cathelicidin, hCAP-18, is processed to the antimicrobial peptide LL-37 by extracellular cleavage with proteinase 3," *Blood,* vol. 97, no. 12, pp. 3951-3959, 2001, doi: 10.1182/blood.V97.12.3951.
[161] U. H. N. Dürr, U. S. Sudheendra, and A. Ramamoorthy, "LL-37, the only human member of the cathelicidin family of antimicrobial peptides," *Biochimica et Biophysica Acta (BBA) - Biomembranes,* vol. 1758, no. 9, pp. 1408-1425, 2006/09/01/ 2006, doi: https://doi.org/10.1016/j.bbamem.2006.03.030.
[162] M. Murakami, B. Lopez-Garcia, M. Braff, R. A. Dorschner, and R. L. Gallo, "Postsecretory processing generates multiple cathelicidins for enhanced topical antimicrobial defense," *The Journal of Immunology,* vol. 172, no. 5, pp. 3070-3077, 2004.
[163] A. M. van der Does *et al.*, "LL-37 Directs Macrophage Differentiation toward Macrophages with a Proinflammatory Signature," *The Journal of Immunology,* vol. 185, no. 3, p. 1442, 2010, doi: 10.4049/jimmunol.1000376.

[164] Y. Glucksam-Galnoy *et al.*, "The Bacterial Quorum-Sensing Signal Molecule N-3-Oxo-Dodecanoyl-l-Homoserine Lactone Reciprocally Modulates Pro- and Anti-Inflammatory Cytokines in Activated Macrophages," *The Journal of Immunology,* p. 1300368, 2013, doi: 10.4049/jimmunol.1300368.
[165] L. Perié, J. Aru, P. Kourilsky, and J.-J. Slotine, "Does a quorum sensing mechanism direct the behavior of immune cells?," *Comptes Rendus Biologies,* vol. 336, no. 1, pp. 13-16, 2013/01/01/ 2013, doi: https://doi.org/10.1016/j.crvi.2013.01.006.
[166] S. Al-Adwani *et al.*, "Studies on citrullinated LL-37: detection in human airways, antibacterial effects and biophysical properties," (in eng), no. 2045-2322 (Electronic).
[167] M. F. Salamah *et al.*, "The endogenous antimicrobial cathelicidin LL37 induces platelet activation and augments thrombus formation," (in eng), no. 2473-9537 (Electronic).
[168] M. Schwaab, A. Gurr, A. Neumann, S. Dazert, and A. Minovi, "Human antimicrobial proteins in ear wax," (in eng), *Eur J Clin Microbiol Infect Dis,* vol. 30, no. 8, pp. 997-1004, Aug 2011, doi: 10.1007/s10096-011-1185-2.
[169] S. B. Coffelt *et al.*, "Ovarian cancers overexpress the antimicrobial protein hCAP-18 and its derivative LL-37 increases ovarian cancer cell proliferation and invasion," *Int. J. Cancer,* vol. 122, no. 5, pp. 1030-1039, 2008/03/01 2008, doi: 10.1002/ijc.23186.
[170] R. Lande *et al.*, "Generation of Monoclonal Antibodies Specific for Native LL37 and Citrullinated LL37 That Discriminate the Two LL37 Forms in the Skin and Circulation of Cutaneous/Systemic Lupus Erythematosus and Rheumatoid Arthritis Patients," (in eng), *Antibodies (Basel, Switzerland),* vol. 9, no. 2, p. 14, 2020, doi: 10.3390/antib9020014.
[171] S. Sakamoto *et al.*, "Enzyme-linked immunosorbent assay for the quantitative/qualitative analysis of plant secondary metabolites," (in eng), *Journal of natural medicines,* vol. 72, no. 1, pp. 32-42, 2018, doi: 10.1007/s11418-017-1144-z.
[172] J. Litwin, "Standardization of human diploid fibroblast cultivation: trypsinization procedure," (in eng), *Applied microbiology,* vol. 21, no. 2, pp. 169-174, 1971, doi: 10.1128/am.21.2.169-174.1971.
[173] A. B. Nowakowski, W. J. Wobig, and D. H. Petering, "Native SDS-PAGE: high resolution electrophoretic separation of proteins with retention of native properties including bound metal ions," (in eng), *Metallomics : integrated biometal science,* vol. 6, no. 5, pp. 1068-1078, 2014, doi: 10.1039/c4mt00033a.
[174] G. Bergsson *et al.*, "LL-37 Complexation with Glycosaminoglycans in Cystic Fibrosis Lungs Inhibits Antimicrobial Activity, Which Can Be Restored by Hypertonic Saline," *The Journal of Immunology,* vol. 183, no. 1, p. 543, 2009, doi: 10.4049/jimmunol.0803959.
[175] Y. Wang, B. Agerberth, A. Löthgren, A. Almstedt, and J. Johansson, "Apolipoprotein A-I Binds and Inhibits the Human Antibacterial/Cytotoxic Peptide LL-37*," *Journal of Biological Chemistry,* vol. 273, no. 50, pp. 33115-33118, 1998/12/11/ 1998, doi: https://doi.org/10.1074/jbc.273.50.33115.
[176] R. Bucki, F. J. Byfield, and P. A. Janmey, "Release of the antimicrobial peptide LL-37 from DNA/F-actin bundles in cystic fibrosis sputum," *European Respiratory Journal,* vol. 29, no. 4, p. 624, 2007, doi: 10.1183/09031936.00080806.
[177] T. Mahmood and P.-C. Yang, "Western blot: technique, theory, and trouble shooting," (in eng), *North American journal of medical sciences,* vol. 4, no. 9, pp. 429-434, 2012, doi: 10.4103/1947-2714.100998.

[178] A. Aidoukovitch *et al.*, "Antimicrobial peptide LL-37 and its pro-form, hCAP18, in desquamated epithelial cells of human whole saliva," (in eng), no. 1600-0722 (Electronic).
[179] E. Bankell *et al.*, "The antimicrobial peptide LL-37 triggers release of apoptosis-inducing factor and shows direct effects on mitochondria," *Biochemistry and Biophysics Reports,* vol. 29, p. 101192, 2022/03/01/ 2022, doi: https://doi.org/10.1016/j.bbrep.2021.101192.
[180] A. Girnita, H. Zheng, A. Grönberg, L. Girnita, and M. Ståhle, "Identification of the cathelicidin peptide LL-37 as agonist for the type I insulin-like growth factor receptor," *Oncogene,* vol. 31, no. 3, pp. 352-365, 2012/01/01 2012, doi: 10.1038/onc.2011.239.
[181] M. Hemshekhar, K.-Y. G. Choi, and N. Mookherjee, "Host Defense Peptide LL-37-Mediated Chemoattractant Properties, but Not Anti-Inflammatory Cytokine IL-1RA Production, Is Selectively Controlled by Cdc42 Rho GTPase via G Protein-Coupled Receptors and JNK Mitogen-Activated Protein Kinase," *Frontiers in immunology*, vol. 9*,* p. 1871doi: 10.3389/fimmu.2018.01871.
[182] Z. Oren, J. C. Lerman, G. H. Gudmundsson, B. Agerberth, and Y. Shai, "Structure and organization of the human antimicrobial peptide LL-37 in phospholipid membranes: relevance to the molecular basis for its non-cell-selective activity," *Biochemical Journal,* vol. 341, no. 3, pp. 501-513, 1999, doi: 10.1042/bj3410501.
[183] Y. Li, X. Li, H. Li, O. Lockridge, and G. Wang, "A novel method for purifying recombinant human host defense cathelicidin LL-37 by utilizing its inherent property of aggregation," *Protein Expression and Purification,* vol. 54, no. 1, pp. 157-165, 2007/07/01/ 2007, doi: https://doi.org/10.1016/j.pep.2007.02.003.
[184] E. Y. Lee *et al.*, "Helical antimicrobial peptides assemble into protofibril scaffolds that present ordered dsDNA to TLR9," *Nature Communications,* vol. 10, no. 1, p. 1012, 2019/03/04 2019, doi: 10.1038/s41467-019-08868-w.
[185] Y. Engelberg and M. Landau, "The Human LL-37(17-29) antimicrobial peptide reveals a functional supramolecular structure," *Nature Communications,* vol. 11, no. 1, p. 3894, 2020/08/04 2020, doi: 10.1038/s41467-020-17736-x.
[186] A. Diaz Perez, K. Kougl, T. W. Vasicek, R. Liyanage, J. Lay, and J. A. Stenken, "Microdialysis Sampling of Quorum Sensing Homoserine Lactones during Biofilm Formation," (in eng), *Anal Chem,* vol. 91, no. 6, pp. 3964-3970, Mar 19 2019, doi: 10.1021/acs.analchem.8b05168.
[187] A. Duplantier and M. van Hoek, "The Human Cathelicidin Antimicrobial Peptide LL-37 as a Potential Treatment for Polymicrobial Infected Wounds," *Frontiers in Immunology,* 10.3389/fimmu.2013.00143 vol. 4, p. 143, 2013.
[188] Y. Al Tall, A. Abualhaijaa, M. Alsaggar, A. Almaaytah, M. Masadeh, and K. H. Alzoubi, "Design and characterization of a new hybrid peptide from LL-37 and BMAP-27," (in eng), *Infect. Drug Resist.,* vol. 12, pp. 1035-1045, 2019, doi: 10.2147/IDR.S199473.
[189] A. Valverde, *EU Guidelines on Method Validation and Quality Control Procedures for Pesticide Residues Analysis in Food and Feed*. 2015.
[190] J. Ma and D. Xia, "The use of blue native PAGE in the evaluation of membrane protein aggregation states for crystallization," *Journal of Applied Crystallography,* vol. 41, no. 6, pp. 1150-1160, 12/01 2008.
[191] W. Wang, "Protein aggregation and its inhibition in biopharmaceutics," *International Journal of Pharmaceutics,* vol. 289, no. 1, pp. 1-30, 2005/01/31/ 2005, doi: https://doi.org/10.1016/j.ijpharm.2004.11.014.

[192] J. Chen, Y. Liu Y Fau - Wang, H. Wang Y Fau - Ding, Z. Ding H Fau - Su, and Z. Su, "Different effects of L-arginine on protein refolding: suppressing aggregates of hydrophobic interaction, not covalent binding," (in eng), no. 1520-6033 (Electronic).
[193] C. Lange and R. Rudolph, "Suppression of Protein Aggregation by L-Arginine," *Current Pharmaceutical Biotechnology,* vol. 10, no. 4, pp. 408-414, 2009, doi: http://dx.doi.org/10.2174/138920109788488851.
[194] E. Hackl, J. Darkwah, G. Smith, and I. Ermolina, "Effect of Arginine on the Aggregation of Protein in Freeze-Dried Formulations Containing Sugars and Polyol: 1-Formulation Development," (in eng), no. 1530-9932 (Electronic).
[195] I. Seifert, A. Bregolin, D. Fissore, and W. Friess, "The Influence of Arginine and Counter-Ions: Antibody Stability during Freeze-Drying," (in eng), no. 1520-6017 (Electronic).
[196] H. Maity, A. O'Dell C Fau - Srivastava, J. Srivastava A Fau - Goldstein, and J. Goldstein, "Effects of arginine on photostability and thermal stability of IgG1 monoclonal antibodies," (in eng), no. 1873-4316 (Electronic).
[197] A. Das and C. Mukhopadhyay, "Urea-Mediated Protein Denaturation: A Consensus View," *The Journal of Physical Chemistry B,* vol. 113, no. 38, pp. 12816-12824, 2009/09/24 2009, doi: 10.1021/jp906350s.
[198] L. Hua, R. Zhou, D. Thirumalai, and B. J. Berne, "Urea denaturation by stronger dispersion interactions with proteins than water implies a 2-stage unfolding," *Proceedings of the National Academy of Sciences,* vol. 105, no. 44, pp. 16928-16933, 2008/11/04 2008, doi: 10.1073/pnas.0808427105.
[199] C. N. Pace, "[14] Determination and analysis of urea and guanidine hydrochloride denaturation curves," in *Methods in enzymology*, vol. 131: Elsevier, 1986, pp. 266-280.
[200] C. Tanford, "Protein denaturation: Part C. Theoretical models for the mechanism of denaturation," *Advances in protein chemistry,* vol. 24, pp. 1-95, 1970.
[201] D. O. V. Alonso and K. A. Dill, "Solvent denaturation and stabilization of globular proteins," *Biochemistry,* vol. 30, no. 24, pp. 5974-5985, 1991.
[202] J. M. Scholtz, D. Barrick, E. J. York, J. M. Stewart, and R. L. Baldwin, "Urea unfolding of peptide helices as a model for interpreting protein unfolding," *Proceedings of the National Academy of Sciences,* vol. 92, no. 1, pp. 185-189, 1995.
[203] G. I. Makhatadze, "Thermodynamics of protein interactions with urea and guanidinium hydrochloride," vol. 103, ed: ACS Publications, 1999, pp. 4781-4785.
[204] S. Ja, "The stability of hydrogen-bonded peptide structures in aqueous solution," *Comptes rendus des travaux du Laboratoire Carlsberg. Serie chimique,* vol. 29, no. 14-15, pp. 230-259, 1955.
[205] K. Lim Woon, J. Rösgen, and S. W. Englander, "Urea, but not guanidinium, destabilizes proteins by forming hydrogen bonds to the peptide group," *Proceedings of the National Academy of Sciences,* vol. 106, no. 8, pp. 2595-2600, 2009/02/24 2009, doi: 10.1073/pnas.0812588106.
[206] Z. Lai, J. McCulloch, H. A. Lashuel, and J. W. Kelly, "Guanidine Hydrochloride-Induced Denaturation and Refolding of Transthyretin Exhibits a Marked Hysteresis: Equilibria with High Kinetic Barriers," *Biochemistry,* vol. 36, no. 33, pp. 10230-10239, 1997/08/01 1997, doi: 10.1021/bi963195p.

[207] M. A. Qasim and M. Taha, "Investigation of the mechanism of protein denaturation by guanidine hydrochloride-induced dissociation of inhibitor-protease complexes," (in eng), no. 1875-5305 (Electronic).
[208] A. Baig Ma Fau - Salahuddin and A. Salahuddin, "Occurrence and characterization of stable intermediate state(s) in the unfolding of ovomucoid by guanidine hydrochloride," (in eng), no. 0264-6021 (Print).
[209] A. T. Iavarone, J. C. Jurchen, and E. R. Williams, "Supercharged Protein and Peptide Ions Formed by Electrospray Ionization," *Analytical Chemistry,* vol. 73, no. 7, pp. 1455-1460, 2001/04/01 2001, doi: 10.1021/ac001251t.
[210] M. Nshanian, R. Lakshmanan, H. Chen, R. R. Ogorzalek Loo, and J. A. Loo, "Enhancing Sensitivity of Liquid Chromatography-Mass Spectrometry of Peptides and Proteins Using Supercharging Agents," (in eng), *International journal of mass spectrometry,* vol. 427, pp. 157-164, 2018, doi: 10.1016/j.ijms.2017.12.006.
[211] R. Gomez-Sanchez *et al.*, "Maintaining a High-Quality Screening Collection: The GSK Experience," *SLAS Discovery,* vol. 26, no. 8, pp. 1065-1070, 2021/09/01/ 2021, doi: https://doi.org/10.1177/24725552211017526.
[212] D. Strzelecka, S. W. Holman, and C. E. Eyers, "Evaluation of dimethyl sulfoxide (DMSO) as a mobile phase additive during top 3 label-free quantitative proteomics," (in eng), *International journal of mass spectrometry,* vol. 391, pp. 157-160, 2015, doi: 10.1016/j.ijms.2015.07.004.
[213] R. Kong, "17 - LC/MS Application in High-Throughput ADME Screen," in *Separation Science and Technology*, vol. 6, S. Ahuja and M. W. Dong Eds.: Academic Press, 2005, pp. 413-446.
[214] H. A. Mowafy, F. K. Alanazi, and G. M. El Maghraby, "Development and validation of an HPLC-UV method for the quantification of carbamazepine in rabbit plasma," (in eng), *Saudi pharmaceutical journal : SPJ : the official publication of the Saudi Pharmaceutical Society,* vol. 20, no. 1, pp. 29-34, 2012/01// 2012, doi: 10.1016/j.jsps.2011.04.003.
[215] M. Jemal, S. Rao, M. Gatz, and D. Whigan, "Liquid chromatography-tandem mass spectrometric quantitative determination of the HIV protease inhibitor atazanavir (BMS-232632) in human peripheral blood mononuclear cells (PBMC): practical approaches to PBMC preparation and PBMC assay design for high-throughput analysis," *Journal of chromatography. B, Analytical technologies in the biomedical and life sciences,* vol. 795, no. 2, pp. 273-89, 10/3/pubmed

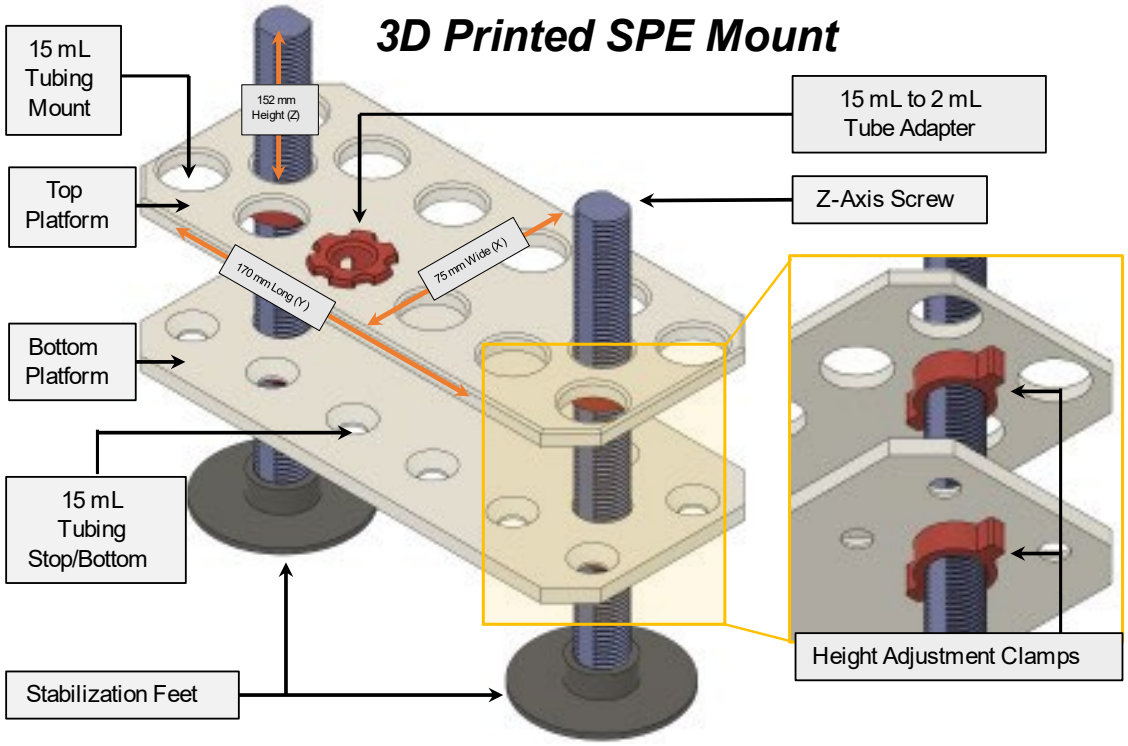
6/21/medline

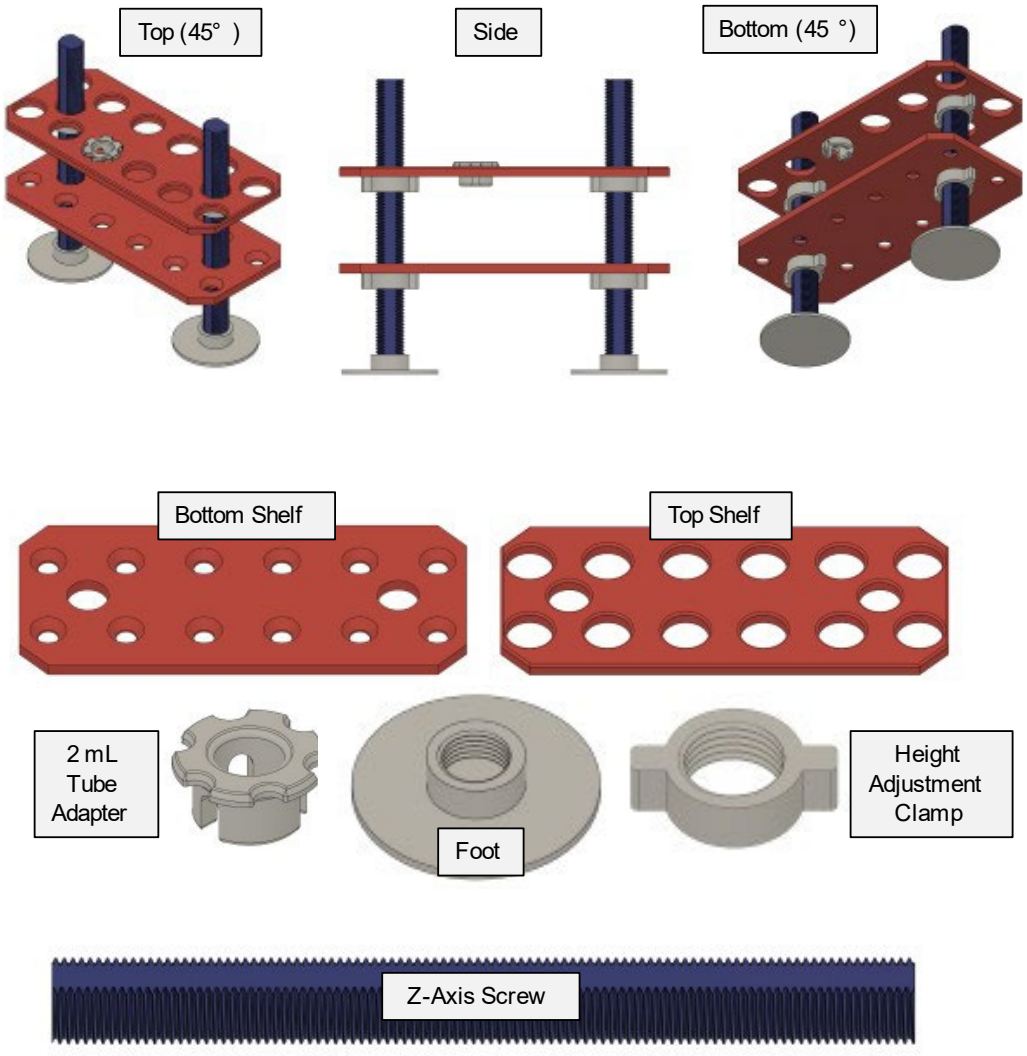
10/3/entrez 2003.
[216] S. Siddiquee and A. M. S. Shafwanah, "10 - Toxicology and Analytical Methods for the Analysis of Allura Red (E129) in Food and Beverage Products: A Current Perspective," in *Safety Issues in Beverage Production*, A. M. Grumezescu and A. M. Holban Eds.: Academic Press, 2020, pp. 335-357.
[217] D. K. Kulal, P. C. Loni, C. Dcosta, S. Some, and P. K. Kalambate, "Chapter 19 - Cyanobacteria: as a promising candidate for heavy-metals removal," in *Advances in Cyanobacterial Biology*, P. K. Singh, A. Kumar, V. K. Singh, and A. K. Shrivastava Eds.: Academic Press, 2020, pp. 291-300.
[218] Y. Al Tall, A. Abualhaijaa, M. Alsaggar, A. Almaaytah, M. Masadeh, and K. H. Alzoubi, "Design and characterization of a new hybrid peptide from LL-37 and BMAP-27," (in

eng), *Infection and drug resistance,* vol. 12, pp. 1035-1045, 2019, doi: 10.2147/IDR.S199473.
[219] N. Fontanals, R. M. Marcé, and F. Borrull, "Materials for Solid-Phase Extraction of Organic Compounds," *Separations,* vol. 6, no. 4, 2019, doi: 10.3390/separations6040056.
[220] S. Dugheri *et al.*, "A review of micro-solid-phase extraction techniques and devices applied in sample pretreatment coupled with chromatographic analysis," (in English), *Acta Chromatographica Acta Chromatographica,* vol. 33, no. 2, pp. 99-111, 19 Mar. 2021 2021, doi: 10.1556/1326.2020.00790.
[221] A. Diaz Perez, K. Kougl, T. W. Vasicek, R. Liyanage, J. Lay, and J. A.-O. Stenken, "Microdialysis Sampling of Quorum Sensing Homoserine Lactones during Biofilm Formation," (in eng), no. 1520-6882 (Electronic).
[222] M. L. Vasil, "Pseudomonas aeruginosa: biology, mechanisms of virulence, epidemiology," (in eng), no. 0022-3476 (Print).
[223] E. Stokvis, J. H. Rosing H Fau - Beijnen, and J. H. Beijnen, "Stable isotopically labeled internal standards in quantitative bioanalysis using liquid chromatography/mass spectrometry: necessity or not?," (in eng), no. 0951-4198 (Print).
[224] E. Stokvis, L. Rosing H Fau - López-Lázaro, J. H. M. López-Lázaro L Fau - Schellens, J. H. Schellens Jh Fau - Beijnen, and J. H. Beijnen, "Switching from an analogous to a stable isotopically labeled internal standard for the LC-MS/MS quantitation of the novel anticancer drug Kahalalide F significantly improves assay performance," (in eng), no. 0269-3879 (Print).
[225] D. Remane, M. R. Wissenbach Dk Fau - Meyer, H. H. Meyer Mr Fau - Maurer, and H. H. Maurer, "Systematic investigation of ion suppression and enhancement effects of fourteen stable-isotope-labeled internal standards by their native analogues using atmospheric-pressure chemical ionization and electrospray ionization and the relevance for multi-analyte liquid chromatographic/mass spectrometric procedures," (in eng), no. 1097-0231 (Electronic).

7 Appendix

7.1 3D Printed SPE Mount Specifications




Top (45°)
Side
Bottom (45 °)
Bottom Shelf
Top Shelf
2 mL Tube Adapter
Foot
Height Adjustment Clamp
Z-Axis Screw

7.2 Copyright

ELSEVIER LICENSE
TERMS AND CONDITIONS

Jun 27, 2022

This Agreement between Victoria Hunter ("You") and Elsevier ("Elsevier") consists of your license details and thewang terms and conditions provided by Elsevier and Copyright Clearance Center.

License Number

5283280436950

License date

Apr 06, 2022

Licensed Content Publisher

Elsevier

Licensed Content Publication

Biochimica et Biophysica Acta (BBA) - Biomembranes

Licensed Content Title

High-quality 3D structures shine light on antibacterial, anti-biofilm and antiviral activities of human cathelicidin LL-37 and its fragments

Licensed Content Author

Guangshun Wang,Biswajit Mishra,Raquel F. Epand,Richard M. Epand

Licensed Content Date

Sep 1, 2014

Licensed Content Volume

1838

Licensed Content Issue

9

Licensed Content Pages

13

Start Page

2160

End Page

2172

Type of Use

reuse in a thesis/dissertation

Portion

figures/tables/illustrations

Number of figures/tables/illustrations

1

Format

electronic

Are you the author of this Elsevier article?

No

Will you be translating?

No

Title

Senior Graduate Assistant

Institution name

University of Arkansas

Expected presentation date

Jul 2022

Portions

Figure 3 A only

Requestor Location

Victoria Hunter
1010 S Sherman Ave

FAYETTEVILLE, AR 72701
United States
Attn: Victoria Hunter

Publisher Tax ID

98-0397604

Total

0.00 USD

Terms and Conditions

INTRODUCTION

1. The publisher for this copyrighted material is Elsevier. By clicking "accept" in connection with completing this licensing transaction, you agree that the following terms and conditions apply to this transaction (along with the Billing and Payment terms and conditions established by Copyright Clearance Center, Inc. ("CCC"), at the time that you opened your Rightslink account and that are available at any time at http://myaccount.copyright.com).

GENERAL TERMS

2. Elsevier hereby grants you permission to reproduce the aforementioned material subject to the terms and conditions indicated.

3. Acknowledgement: If any part of the material to be used (for example, figures) has appeared in our publication with credit or acknowledgement to another source, permission must also be sought from that source. If such permission is not obtained then that material may not be included in your publication/copies. Suitable acknowledgement to the source must be made, either as a footnote or in a reference list at the end of your publication, as follows:

"Reprinted from Publication title, Vol /edition number, Author(s), Title of article / title of chapter, Pages No., Copyright (Year), with permission from Elsevier [OR APPLICABLE SOCIETY COPYRIGHT OWNER]." Also Lancet special credit - "Reprinted from The Lancet, Vol. number, Author(s), Title of article, Pages No., Copyright (Year), with permission from Elsevier."

4. Reproduction of this material is confined to the purpose and/or media for which permission is hereby given.

5. Altering/Modifying Material: Not Permitted. However figures and illustrations may be altered/adapted minimally to serve your work. Any other abbreviations, additions, deletions and/or any other alterations shall be made only with prior written authorization of Elsevier Ltd. (Please contact Elsevier's permissions helpdesk here). No modifications can be made to any Lancet figures/tables and they must be reproduced in full.

6. If the permission fee for the requested use of our material is waived in this instance, please be advised that your future requests for Elsevier materials may attract a fee.

7. Reservation of Rights: Publisher reserves all rights not specifically granted in the combination of (i) the license details provided by you and accepted in the course of this licensing transaction, (ii) these terms and conditions and (iii) CCC's Billing and Payment terms and conditions.

8. License Contingent Upon Payment: While you may exercise the rights licensed immediately upon issuance of the license at the end of the licensing process for the transaction, provided that you have disclosed complete and accurate details of your proposed use, no license is finally effective unless and until full payment is received from you (either by publisher or by CCC) as provided in CCC's Billing and Payment terms and conditions. If full payment is not received on a timely basis, then any license preliminarily granted shall be deemed automatically revoked and shall be void as if never granted. Further, in the event that you breach any of these terms and conditions or any of CCC's Billing and Payment terms and conditions, the license is automatically revoked and shall be void as if never granted. Use of materials as described in a revoked license, as well as any use of the materials beyond the scope of an unrevoked license, may constitute copyright infringement and publisher reserves the right to take any and all action to protect its copyright in the materials.

9. Warranties: Publisher makes no representations or warranties with respect to the licensed material.

10. Indemnity: You hereby indemnify and agree to hold harmless publisher and CCC, and their respective officers, directors, employees and agents, from and against any and all claims arising out of your use of the licensed material other than as specifically authorized pursuant to this license.

11. No Transfer of License: This license is personal to you and may not be sublicensed, assigned, or transferred by you to any other person without publisher's written permission.

12. No Amendment Except in Writing: This license may not be amended except in a writing signed by both parties (or, in the case of publisher, by CCC on publisher's behalf).

13. Objection to Contrary Terms: Publisher hereby objects to any terms contained in any purchase order, acknowledgment, check endorsement or other writing prepared by you, which terms are inconsistent with these terms and conditions or CCC's Billing and Payment terms and conditions. These terms and conditions, together with CCC's Billing and Payment terms and conditions (which are incorporated herein), comprise the entire agreement between you and publisher (and CCC) concerning this licensing transaction. In the event of any conflict between your obligations established by these terms and conditions and those established by CCC's Billing and Payment terms and conditions, these terms and conditions shall control.

14. Revocation: Elsevier or Copyright Clearance Center may deny the permissions described in this License at their sole discretion, for any reason or no reason, with a full refund payable to you. Notice of such denial will be made using the contact information provided by you. Failure to receive such notice will not alter or invalidate the denial. In no event will Elsevier or Copyright Clearance Center be responsible or liable for any costs, expenses or damage incurred by you as a result of a denial of your permission request, other than a refund of the amount(s) paid by you to Elsevier and/or Copyright Clearance Center for denied permissions.

LIMITED LICENSE

The following terms and conditions apply only to specific license types:

15. **Translation**: This permission is granted for non-exclusive world **English** rights only unless your license was granted for translation rights. If you licensed translation rights you may only translate this content into the languages you requested. A professional translator must perform all translations and reproduce the content word for word preserving the integrity of the article.

16. **Posting licensed content on any Website**: The following terms and conditions apply as follows: Licensing material from an Elsevier journal: All content posted to the web site must maintain the copyright information line on the bottom of each image; A hyper-text must be included to the Homepage of the journal from which you are licensing at http://www.sciencedirect.com/science/journal/xxxxx or the Elsevier homepage for books at http://www.elsevier.com; Central Storage: This license does not include permission for a

scanned version of the material to be stored in a central repository such as that provided by Heron/XanEdu.

Licensing material from an Elsevier book: A hyper-text link must be included to the Elsevier homepage at http://www.elsevier.com . All content posted to the web site must maintain the copyright information line on the bottom of each image.

Posting licensed content on Electronic reserve: In addition to the above the following clauses are applicable: The web site must be password-protected and made available only to bona fide students registered on a relevant course. This permission is granted for 1 year only. You may obtain a new license for future website posting.

17. **For journal authors:** the following clauses are applicable in addition to the above:

Preprints:

A preprint is an author's own write-up of research results and analysis, it has not been peer-reviewed, nor has it had any other value added to it by a publisher (such as formatting, copyright, technical enhancement etc.).

Authors can share their preprints anywhere at any time. Preprints should not be added to or enhanced in any way in order to appear more like, or to substitute for, the final versions of articles however authors can update their preprints on arXiv or RePEc with their Accepted Author Manuscript (see below).

If accepted for publication, we encourage authors to link from the preprint to their formal publication via its DOI. Millions of researchers have access to the formal publications on ScienceDirect, and so links will help users to find, access, cite and use the best available version. Please note that Cell Press, The Lancet and some society-owned have different preprint policies. Information on these policies is available on the journal homepage.

Accepted Author Manuscripts: An accepted author manuscript is the manuscript of an article that has been accepted for publication and which typically includes author-incorporated changes suggested during submission, peer review and editor-author communications.

Authors can share their accepted author manuscript:

- immediately
    - via their non-commercial person homepage or blog
    - by updating a preprint in arXiv or RePEc with the accepted manuscript
    - via their research institute or institutional repository for internal institutional uses or as part of an invitation-only research collaboration work-group
    - directly by providing copies to their students or to research collaborators for their personal use

    - for private scholarly sharing as part of an invitation-only work group on commercial sites with which Elsevier has an agreement
- After the embargo period
    - via non-commercial hosting platforms such as their institutional repository
    - via commercial sites with which Elsevier has an agreement

In all cases accepted manuscripts should:

- link to the formal publication via its DOI
- bear a CC-BY-NC-ND license - this is easy to do
- if aggregated with other manuscripts, for example in a repository or other site, be shared in alignment with our hosting policy not be added to or enhanced in any way to appear more like, or to substitute for, the published journal article.

Published journal article (JPA): A published journal article (PJA) is the definitive final record of published research that appears or will appear in the journal and embodies all value-adding publishing activities including peer review co-ordination, copy-editing, formatting, (if relevant) pagination and online enrichment.

Policies for sharing publishing journal articles differ for subscription and gold open access articles:

Subscription Articles: If you are an author, please share a link to your article rather than the full-text. Millions of researchers have access to the formal publications on ScienceDirect, and so links will help your users to find, access, cite, and use the best available version.

Theses and dissertations which contain embedded PJAs as part of the formal submission can be posted publicly by the awarding institution with DOI links back to the formal publications on ScienceDirect.

If you are affiliated with a library that subscribes to ScienceDirect you have additional private sharing rights for others' research accessed under that agreement. This includes use for classroom teaching and internal training at the institution (including use in course packs and courseware programs), and inclusion of the article for grant funding purposes.

Gold Open Access Articles: May be shared according to the author-selected end-user license and should contain a CrossMark logo, the end user license, and a DOI link to the formal publication on ScienceDirect.

Please refer to Elsevier's posting policy for further information.

18. **For book authors** the following clauses are applicable in addition to the above: Authors are permitted to place a brief summary of their work online only. You are not allowed to download and post the published electronic version of your chapter, nor may you scan the printed edition to create an electronic version. **Posting to a repository:** Authors are permitted to post a summary of their chapter only in their institution's repository.

19. **Thesis/Dissertation**: If your license is for use in a thesis/dissertation your thesis may be submitted to your institution in either print or electronic form. Should your thesis be published commercially, please reapply for permission. These requirements include permission for the Library and Archives of Canada to supply single copies, on demand, of the complete thesis and include permission for Proquest/UMI to supply single copies, on demand, of the complete thesis. Should your thesis be published commercially, please reapply for permission. Theses and dissertations which contain embedded PJAs as part of the formal submission can be posted publicly by the awarding institution with DOI links back to the formal publications on ScienceDirect.

Elsevier Open Access Terms and Conditions

You can publish open access with Elsevier in hundreds of open access journals or in nearly 2000 established subscription journals that support open access publishing. Permitted third party re-use of these open access articles is defined by the author's choice of Creative Commons user license. See our open access license policy for more information.

Terms & Conditions applicable to all Open Access articles published with Elsevier:

Any reuse of the article must not represent the author as endorsing the adaptation of the article nor should the article be modified in such a way as to damage the author's honour or reputation. If any changes have been made, such changes must be clearly indicated.

The author(s) must be appropriately credited and we ask that you include the end user license and a DOI link to the formal publication on ScienceDirect.

If any part of the material to be used (for example, figures) has appeared in our publication with credit or acknowledgement to another source it is the responsibility of the user to ensure their reuse complies with the terms and conditions determined by the rights holder.

Additional Terms & Conditions applicable to each Creative Commons user license:

CC BY: The CC-BY license allows users to copy, to create extracts, abstracts and new works from the Article, to alter and revise the Article and to make commercial use of the Article (including reuse and/or resale of the Article by commercial entities), provided the user gives appropriate credit (with a link to the formal publication through the relevant DOI), provides a link to the license, indicates if changes were made and the licensor is not represented as endorsing the use made of the work. The full details of the license are available at http://creativecommons.org/licenses/by/4.0.

CC BY NC SA: The CC BY-NC-SA license allows users to copy, to create extracts, abstracts and new works from the Article, to alter and revise the Article, provided this is not done for commercial purposes, and that the user gives appropriate credit (with a link to the formal publication through the relevant DOI), provides a link to the license, indicates if changes were

---
