REPUBLIC OF TURKEY

ACIBADEM MEHMET ALİ AYDINLAR UNIVERSITY

INSTITUTE OF HEALTH SCIENCES

DEVELOPMENT OF ANTIMICROBIAL CATHETER VIA NOVEL SURFACE CONJUGATION STRATEGIES

İREM SOYHAN

MASTER THESIS

DEPARTMENT OF MEDICAL BIOTECHNOLOGY

SUPERVISOR

Assist. Prof. Özgül Gök

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Assist. Prof. Nihan Ünübol

ISTANBUL 2021

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DECLARATION

I hereby declare that; this thesis has been composed by myself based on the data in line with the scientific rules and ethical principles of responsible conduct of research. All information, data, comments, analyses have been collected and processed through scientific, academic writing style, and literature used have been duly shown by giving reference to the original sources in accordance with the publication ethics. I emphasize that I have not violated any patents and copyrights throughout this study and writing this thesis.

18.06.2021

İrem Soyhan

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LIST OF ABBREVIATIONS AND SYMBOLS

ACN	Acetonitrile
AM	Antimicrobial
AMPs	Antimicrobial peptides
AMR	Antimicrobial resistance
Arg, R	Arginine
ATR/FT-IR	Attenuated total reflection Fourier transform infrared
ATS	Allytrimethoxysilane
BAI	Biomaterial-associated infection
CA°	Contact angle
C-AMPs	Cysteine antimicrobial peptide
CAUTIs	Catheter-associated urinary tract infections
Cys, C	Cysteine
DCM	Dichloromethane
dH_2O	Distilled water
DIC	N,N'-Diisopropylcarbodiimide
DMF	Dimethylformamide
DMPA	2,2-dimethoxy-2-phenylacetophenone
DTT	Dithiothreitol
EMC	Extracellular matrix components
Et_3N	Triethylamine
FA	Formic acid

Fmoc	Fluoren-9-ylmethyloxycarbonyl
GSH	L- Glutathione reduced
HAI	Healthcare-associated infection
LC-MS/MS	Liquid chromatography mass spectrometry
Leu, L	Leucine
Lys, K	Lysine
Mag2	Magainin-2
MIC	Minimal inhibition concentration
MRSA	Methilicin-Resistant Staphylococcus
PDMS	Polydimethylsiloxane
RP-HPLC	Reverse phase high pressure liquid chromatography
SEM-EDS	Scanning electron microscopy- energy dispersion x-ray spectroscopy
SPPS	Solid-phase peptide synthesis
St	Stretching
TEC	Thiol-ene click
TEGDiMA	Triethylene glycol dimethacrylate
TFA	Trifluoroacetic acid
TIS	Triisopropylsilane
TNB	2-nitro-5-thiobenzoic acid

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SUMMARY

Today, 40% of the hospital infections are caused by the urinary tract infections and within this fraction; 80% of them are due to the regularly utilized catheters. Especially in cases where catheter usage is necessary for more than one week, microorganisms can produce biofilm on the surface of the catheter. At this point, antibiotic resistant microorganisms become a very severe thread not only to patients' overall health but also to all people who treated in the hospital, can face with the hospital infection. To decrease this possibility and prevent catheter-associated hospital infections, there have been a growing body of studies being conducted in an interdisciplinary fashion related to medical field. The production of biomaterials which would have the potential to block the binding of the microorganisms on to catheter surface and /or directly kill the microorganisms outstands as an effective method like decorating the surface of catheter with the antimicrobial molecules. However, antibiotic resistance of the bacteria due to the limited applications of these strategies makes already existing antimicrobial treatments ineffective. In this thesis, it is aimed to develop an antimicrobial peptide (AMP) immobilized catheter surface, where the AMPs were developed at School of Medicine at ACU, and a novel conjugation strategy was performed at Nanobiotechnology Center at ACU. Ultimately, it is expected to transfer this strategy to large-scale modification of hospital catheters that are already used in hospitals regularly. By this way, catheter related hospital infections are expected to be decreased with the new generation peptide antibiotics coated catheters. The purpose of this project is to immobilize the new generation antimicrobial peptides, developed based on the peptide antibiotics that are hard or impossible to develop a resistance against, on the surface of the silicone catheters. Hence, the main goal is the obtain more efficient catheter implants than already existing ones by modifying with antimicrobial properties.

Keywords: Antimicrobial Peptides (AMP), Catheter-Associated Urinary Tract Infections (CAUTIs), Peptide Conjugation, Surface Modifications, Thiol-ene Chemistry

ÖZET

Yenilikçi Yüzey Bağlama Stratejileri Kullanılarak Antimikrobiyal Özellikte Kateterlerin Geliştirilmesi

Günümüzde hastane enfeksiyonlarının %40'ını, idrar yolu enfeksiyonları oluşturmaktadır ve bu enfeksiyonların %80'ine ise kateterler neden olmaktadır. Özellikle, bir haftadan daha uzun süre kullanılması zorunlu hallerde, kateter yüzeyinde mikroorganizmalar, biyofilm oluşturabilmektedir. Antibiyotiklere direnç kazanmış mikroorganizmalar, bu aşamadan sonra, sadece, kateteri kullanan hastanın değil, hastane enfeksiyonu olarak tanımlanan ve hastanın tedavi gördüğü hastaneyle başlayıp yeryüzündeki bütün insanlar için yeni bir tehdit kaynağı olmaktadır. Bununla mücadelede, ilk hedef olan kateterlerin, mikroorganizmaların tutunmasını engelleyecek veya doğrudan mikroorganizmaları öldürecek malzemelerden üretilmesi veya yüzeylerinin antimikrobiyal özellikli moleküllerle kaplanması konusunda ciddi çalışmalar mevcuttur. Fakat bu stratejilerin uygulama alanı kısıtlamalarından dolayı bakterilerin geliştirdiği direnç, var olan antimikrobiyal tedavileri etkisiz kılabilmektedir. Bu yüksek lisans tezi kapsamında, ACU Tıp Fakültesi'nde AMP'lerin geliştirildiği ve ACU'daki Nanobiyoteknoloji Merkezi'nde yeni bir konjugasyon stratejisinin uygulandığı antimikrobiyal peptit (AMP) immobilize kateter yüzeyinin geliştirilmesi hedeflenmektedir. Böylelikle yeni nesil peptit antibiyotikler ile kaplanmış kateterlerin, kateter kaynaklı hastane enfeksiyonlarını azaltması amaçlanmaktadır. Projenin amacı, bakteriler tarafından direnç geliştirilmesi olanaksız ya da çok zor olan, doğal peptit antibiyotiklerden esinlenerek geliştirilen yeni nesil AMP'lerin silikon bazlı kateterlerin yüzeyine immobilize edilmesidir. Dolayısıyla, mevcut antimikrobiyal özellikteki kateterlerden daha etkili bir kateter elde edilmesi ana hedef olarak belirlenmiştir.

Anahtar Sözcükler: Antimikrobiyal Peptitler (AMP), Kateter İlişkili Üriner Sistem Enfeksiyonu (Kİ-ÜSE), Peptit Konjügasyonu, Thiol-in Kimyası, Yüzey Modifikasyonları

1. BACKGROUND AND AIM OF THE STUDY

The aim of this thesis is to develop a novel conjugation method for the inhouse designed and synthesized antimicrobial peptides (AMPs) by using a single intermediate molecule as a linker and by comparing the two thiol-ene click (TEC) reactions which are the free-radical addition and the nucleophilic addition methods.

In a previous project, conserved amino acid sequences were designed to mimic the cathecilidin LL-37 and also other AMPs with helical structures (1). Similar to those AMPs designed previously, conserved amino acid sequences synthesized for this study also consist of abundantly arginine (R) and leucine (L). Furthermore, AMP sequence for this thesis has repetitive R residues and for surface immobilization both ends N terminus and C terminus are thiolated separately.

Within the scope of this project, thiolated antimicrobial peptides (C-AMPs) were synthesized, purified, and characterized with HPLC and LC-MS/MS. Later on, in vitro experiments such as cytotoxicity, minimal inhibition concentration (MIC) and hemolytic studies of purified C-AMPs were conducted. To achieve a better immobilization with a single intermediate molecule allytrimethoxysilane (ATS), C-AMPs were immobilized to medical silicone catheter surface via two different thiol-ene click (TEC) chemistry. C-AMP immobilized catheters surfaces were characterized with ATR/FT-IR, contact angle and peptide mapping with SEM-EDS. As a result, it was achieved to obtain more efficient catheter pieces with additional antimicrobial properties by immobilizing C-AMPs on.

2. INTRODUCTION

2.1. Antimicrobial Resistance (AMR)

Antimicrobial Resistance (AMR) is recognized by the World Health Organization (WHO) and has become a prior problem. AMR is a serious problem that imperils efficacy of the drugs against parasite, virus, fungi, and bacteria related diseases. AMR can be caused by the misuse of the antibiotics, overuse of antimicrobial drugs in agriculture and in animal sectors, mutations in microorganisms (2). Since the AMR renders the effectiveness of the antimicrobial drugs, it causes a threat of persistent infection and increases the risk of the spread of.

For fighting against the AMR, antimicrobial peptides (AMPs) can be a promising solution. AMPs are less prone to bacteria gain resistance against. This is because, the pharmacodynamic properties and their working process are different then the common antibiotics (3).

2.2. Antimicrobial (AM) Compounds

2.2.1. AM drugs-antibiotics

As the AMR continues as one of the crucial health crisis the old AM drugs or antibiotics are revised or new ones are designed. In general, antibacterial drugs are classified into two groups based on different type of actions which are the ones are called as bacteriostatic and bactericidal AM drugs. Drugs with the potential of killing the bacteria are in the bactericidal group and the drugs that are inhibit the growth of the bacteria are in the bacteriostatic group. Other classifying the AM agents based on

their type of actions, they can be grouped based on their chemical structures, activity spectrum,mode of action and their source(4). Different AM agents have different properties and last decades the incorporation of these AM agents and the medical devices are significantly increased to fight with the AMR.

2.2.2. AM polymers

While AM polymers consist of the ability to kill or inhibit the growth of the microorganisms. They can either have the antimicrobial activities due to the quaternary nitrogen groups, poly-L-lysine, and halamines in their chemical structure or they can be used as a support to increase the efficacy of the antibiotics (5). Natural polymers like chitosan can exert antimicrobial activity rely on their polymeric features such as molecular weight, chelating capacity, hydrophilicity/hydrophobicity, charge density etc.(6).

2.2.3. AM proteins and peptides

Antimicrobial peptides (AMPs) are the crucial defensive agent of the innate immune system of the living organisms against pathogens. The classification of the discovered AMPs is difficult due to their diversity. For this the simplest classification of the AMPs made according to their secondary structure. The most frequent AMPs that are found in the nature has the α-helices and/or β-sheets in their secondary structures (7).

Generally, AMPs consist of short peptide sequences; less than 100 amino acids, have amphipathic nature, and in general they have net positive charge (8). Since these AMPs have excessive cationic charge, they are rich in cationic lysine (Lys, K) and arginine (Arg, R) amino acid residues (9). The cationic and hydrophobic amino acid

residues provide the AMPs to their amphipathicity which makes AMPs to interact with the bacterial membrane due to its anionic nature (10). The interaction in between the positively charged AMPs and negatively charged bacterial membrane caused by the electrostatic interaction. This electrostatic interaction cause absorption of the AMPs and then insertion of AMPs' hydrophobic residues to the hydrophobic portion bacterial membrane (11). This is known as permeation effect (10,11).

AMPs are also more effective than conventional antibiotics, they have wide-ranging antiviral, antibacterial, and antifungal activities with no toxic effect on the host. They are even effective against antibiotic resistant bacteria and proved to be hard to produce bacterial drug resistant against them (11–13).

2.3. Production of AMPs

The most general classification of the AMPs production can be fall into three groups that are *in vivo* production, cell-free system, and chemical synthesis.

Since AMPs are naturally found and an important part of the innate immune system, one of the procedures that can be used for obtaining the natural AMPs direct isolation from the host through extraction if the desired AMP is already produced by the host. However, this option has limitation and that is the amount of the natural AMPs that is already present within the host (14). Also, for additional research with the site-directed mutagenesis desired changes can be achieved in the amino acid sequences. Although *in vivo* protein expression has the most application, it is restricted to 20 amino acids and proteins that are normally toxic to host such as proteases cannot be produced via this method (15).

Another option to produce the natural AMPs is the cell-free protein production systems. This system consists of the crude cell lysate that is obtained from the growing desired host cell not the host itself unlike the *in vivo* protein expression. The content of the crude cell lysate includes protein synthesis machinery and all the required enzymes as well as the external supply of the essential amino acids, nucleotides, DNA template, messenger RNA (mRNA) (16). The cell-free system has advantages over *in vivo* protein expression system. One of the advantages is the production of the novel proteins through the incorporation of the non-natural or chemically modified amino acids. Although the incorporation of the novel amino acids is the advantage of cell-free system, these amino acids are incompatible with the protein synthesis machinery (ribosomal protein synthesis) (17). The other advantage is the production of the proteins that are toxic to host or proteins that are unstable etc. (18).

The final group in the classification of the production of AMPs is the chemical synthesis which gains popularity through years. Solid-phase peptides synthesis (SPPS) is a good alternative to the cell-based protein synthesis to produce the small AMPs. One of the biggest drawbacks of the cell-based methods is the usage of the chemically modified amino acid which are toxic to living organisms. In cell-based methods especially in *in vivo* only essential amino acid can be used for the synthesis. However, in SPPS this drawback is no longer be an issue. Since there is neither host cell nor natural protein synthesis machinery is used during the SPPS, the incorporation of the unnatural amino acid can be easily achieved (19).

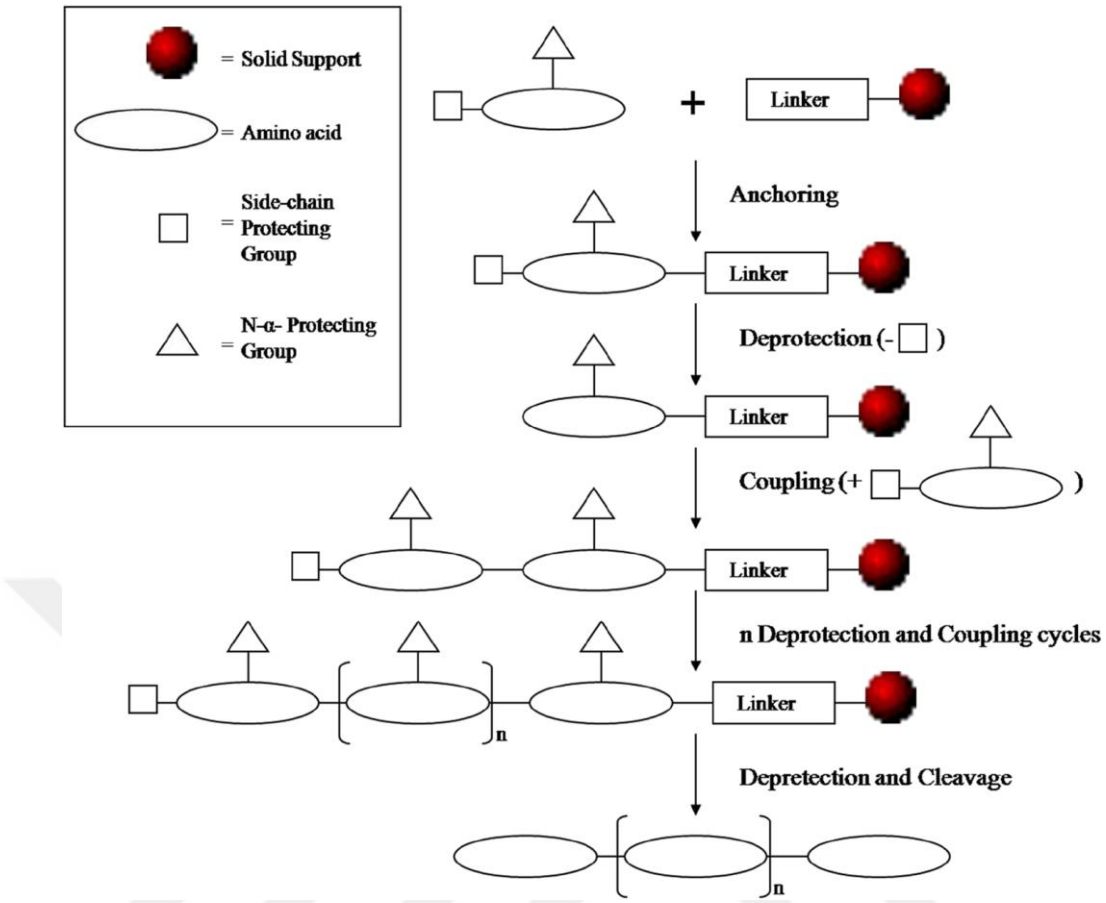

Figure 2.1. Schematic representation of the SPPS method (19).

The SPPS method that is suggested by Merrifield in 1963 has a basic concept of elongating the peptide chain from a solid support through a series of deprotection and coupling cycles for each added amino acid. After the peptide reach to the desired length, it is cleaved from the solid support.

Unlike cell-based peptide synthesis, the peptide elongation direction is C-terminal to N-terminal in SPPS. The reason for this is that the first amino acid is bound to resin through its carboxyl group and in every amino acid coupling there is a deprotection steps that is the removal of the protection group from the amino acid that is going to be coupled to the peptide sequence. Two types of C-terminal can be achieved with SPPS, C-terminal carboxyl end and C-terminal amide end. For the C-terminal amide end peptide synthesis, N-terminal protected amino acid covalently bound to the amino

resin from its carboxyl end to create an amide-linked amino acid and that will eventually produce amide-linked peptide. For the C-terminal acid peptide the proses occur but this time amino acid bound to hydroxyl resin to form ester-linked peptide (19).

Like it is shown in the Figure 1.3.1 the amino acids that are used in the peptide synthesis are coupled with the resin with alpha amino groups (N-α) in their structure and their side chains are protected with various protecting groups temporarily to prevent the side reactions that might occur with the reagents during synthesis. At the beginning of the peptide synthesis, after deciding the desired C-terminal end whether it is amide end or acid end, the carboxyl group of the first amino acid is activated and then coupled to the resin to support the growing peptide chain. When the first amino acid is bound to the resin, filtration process occurs to remove the excess reagent used and the byproducts that are produced during the deprotection step. After filtration the protecting groups of the second N-α protected amino acid removed with deprotection step. Next, again the resin is washed and filtered to remove excess reagents and the byproducts. This cycle is repeated for each N-α protected amino acid addition to the growing peptide chain until the desired sequence is completed. As a final step the synthesized peptide is cleaved from the resin support after removing all the excess reagent and byproducts.

Based on the N-terminal protecting group the features of the peptide synthesis strategy change. There are two the most common protecting groups for the peptide synthesis which are the *tert*-butoxy-carbonyl (Boc) and the other one is the fluoren-9-ylmethyloxycarbonyl (Fmoc). Depending on the synthesis strategy the used resin (Figure 1.3.2) and the reagents are change. Fmoc strategy is preferred more over the Boc strategy. This is because Fmoc protecting group can be removed at milder conditions compered to Boc protecting group (19,20). In this study Fmoc strategy is used for the peptide synthesis.

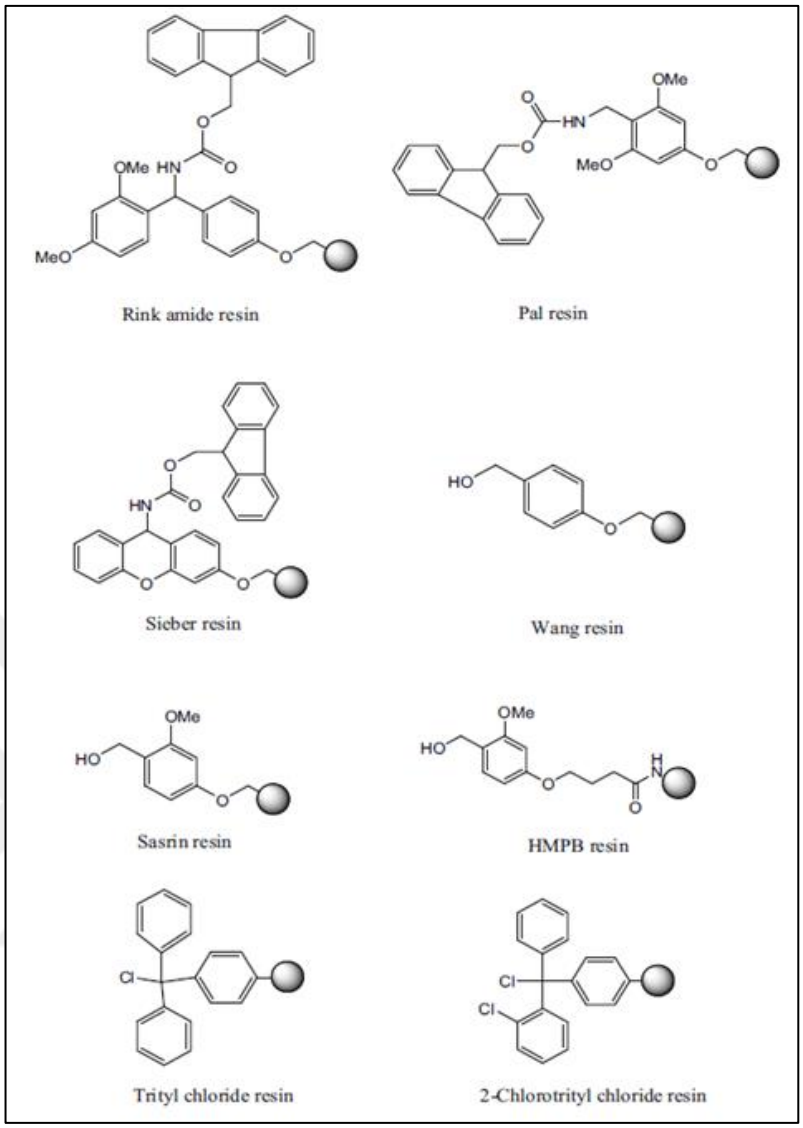

Figure 2.2. Resin types for Fmoc SPPS (19).

2.4. AMP Modified Medical Implants

Over recent decades the need and the usage of the implantable medical devices such as catheters, heart valves, and joint implants etc. increased substantially. Though there is a continuous improvement on the design and production of these medical devices, the risk of developing the healthcare-associated infections (HAIs) or biomaterial-associated infection (BAI) is still a major issue. For combating this major problem, the usage of the conventional antibiotics becomes less effective over time

due to the low concentration on the infection site (21). This situation gets a bigger problem when a biofilm is started to form an/or antibiotic resistant bacteria started to grow on the infection site. To keep the infection under control the removal of the device has become the last resort. Instead of letting for this case to happen alternative solutions needed to be found. AMPs and their application to medical devices has become a good alternative to the conventional antibiotics. With the incorporation of the AMPs, medical devices with antimicrobial properties such as surfaces that kills in contact with microorganisms, surfaces that can release antimicrobials, surfaces with antifouling effects etc. can be produced (22).

2.4.1. Catheter-Associated Urinary Tract Infections (CAUTIs)

For saving patient's lives and improving the therapy the implantable medical devices such as catheters has an important role in the medicine. However, as the implementation duration increases there is a risk of developing an infection while receiving the treatment which is known as the healthcare-associated infection (HAIs) also increases. One of the most common infection types of HAI occurs with the usage of the catheter called as the catheter-associated urinary tract infection (CAUTIs).

Depending on the required treatment and the function of the catheter that the patient needs the catheterization period may vary from a couple days to weeks or even my need to implant catheters permanent. Due to this catheterization period the CAUTIs cause crucial risk factor (23).

The microorganisms that can cause infection start to colonize after adhering to the implanted catheter surface. These infection causing microorganisms can be either the patient's own floral microorganisms or can be come from an exogenous source like from other patients, contaminated other medical devices or even the healthcare personnel.

Among the medical devices that has a rough and hydrophobic surface properties have a higher probability of the bacterial adhesion (24). The obstruction of initial adhesion of the microorganism is an easier and reversible process, however when the microorganisms adhere and with the start to colonize and even aggregates with different species on the surface the hydrophobic medical devices like silicone catheters this process becomes irreversible.

During the urinary catheter insertion procedure, urinary components like organic component accumulate on the catheter surface which result the film formation. The bacteria adhere this formed film on the surface of the catheter and as the time adhered bacteria multiply and excrete its extracellular matrix components (EMCs) which cause the biofilm. While this formed biofilm is important for bacteria to protect itself against antimicrobial agents and the immune system, it also causes CAUTIs to the catheter implanted patients. The most common CAUTIs causing agents are *Escherichia coli* (*E.coli*), *Staphylococcusaureus* (*S.aureus*), *Pseudomonas aeruginosa* (*P. aeruginosa*) etc. (25).

2.4.2. Catheter surface modification of silicone surfaces

To fight and prevent the occurrence of the HAIs investigations about producing medical devices with antimicrobial properties increased. There are several strategies that researches adopt to design and produce antimicrobial biomaterials which can be grouped as the surfaces that coated with antimicrobial agents and surfaces that repel the microbial proteins or microorganism itself (26). Due to the surface roughness and hydrophobicity preventing the initial bacterial adhesion is important. For this one of the approaches to design a non- fouling surface to affect both conformation and the quantity of the absorbed protein such as polyethylene glycol-coated surfaces (27). As a result, the initial adhesion and even the biofilm formation can be prevented.

However, one of the drawback of this approach is that depending on the used non-fouling component it affects the specific bacterial species (28).

The other approach is the surface coating with antimicrobial agents such as biocidal molecules or antibiotics. One of the advantages of this approach is that it provides the direct drug delivery to the implantation site (29). There are various antimicrobial surfaces have been suggested and described in literature such as silver based surfaces (30) or poly(tetralfouroethalene) (PTFE) coated surfaces (31) can be examples. Nevertheless, many of these antimicrobial surfaces are associated with the low efficacy or even cytotoxic effect (32). Due to these limitations surface coating with antibiotics like penicillin, ampicillin, vancomycin etc. has been used (33). Although the usage of the true antibiotics as a coating agent can be good alternative, their effectiveness can only be useful in the spectrum of the chosen antibiotic. Also, there is a high possibility of developing an antibiotic resistance in time (34).When all the disadvantages of different types of antimicrobial surface strategies are considered a good alternative must be found. A good alternative is hard to develop a bacterial resistance against, effective against a broad-spectrum of bacterial species and non-toxic to host itself. AMPs are a meet these criteria and became a good alternative as a surface coating material (35).

2.4.2.1. Thiol-ene chemistry

Thiol-ene click (TEC) reactions are stereospecific means highly specific reactions, efficient and result in high yield, require simple reaction conditions, and generate no by-products. It occurs in between a thiol (R-SH) and an alkene (-C=C-) to forma a thioether (-C-S-C-). The general TEC reaction process starts with the thiyl radical (RS) is generation the from thiol which undergoes to an anti-Markovnikov addition with the alkene to forma a carbon-centered radical. The newly formed carbon-centered radical removes a hydrogen from another thiol to from a thioether product (36).

The initiation step of TEC reaction which is the formation of thiyl radical can be facilitated two ways; one way is the free-radical addition and other way is the Michael addition reactions also known as the nucleophilic addition reaction. Free-radical can be formed via heat, light, or radical initiator.

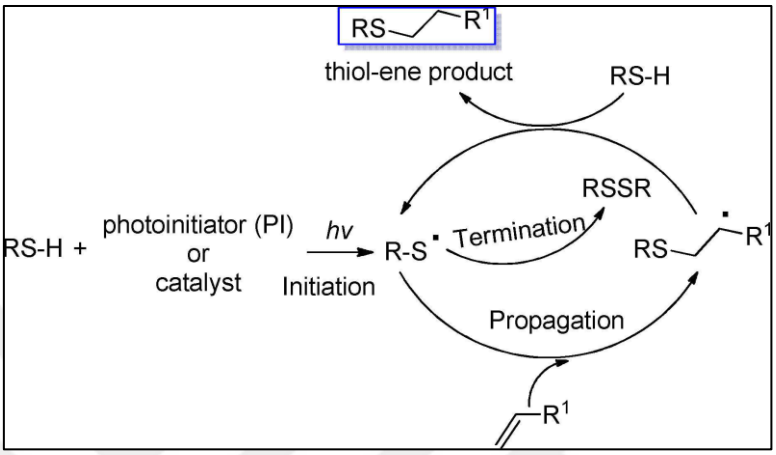

Figure 2.3. Free-radical mechanism of thiol-ene click (TEC) reaction (37).

When the chain initiation step is started with the formation of the thiyl radical, the reaction proceeds to the two-step propagation. In the two-step propagation, the formed thiyl radical directly added to the carbon double bonded alkene to generate a carbon-centered radical which then transferred to the second thiol molecule to form a thiol-ene product with anti-Markovnikov addition orientation while producing a new thiyl radical for the further reaction.

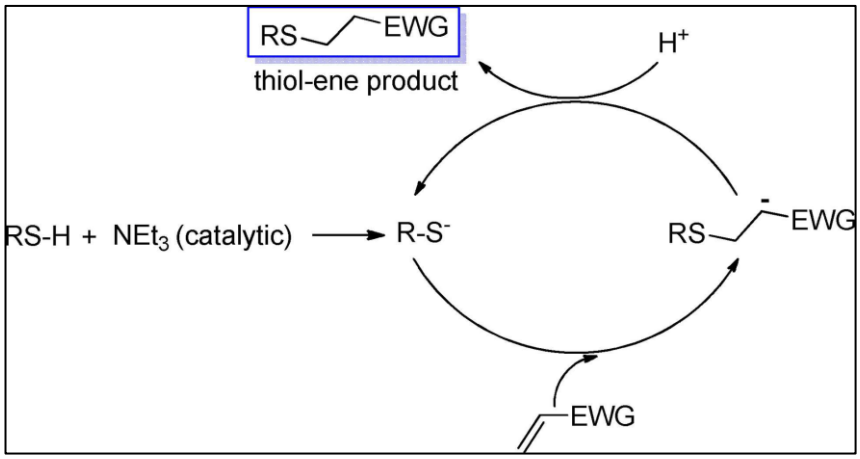

Figure 2.4. Michael-nucleophilic addition pathway of thiol-ene click (TEC) reaction (37).

In the Michael addition also known as the nucleophilic reaction the, a catalytic base deprotonates the thiol and generates the thiyl radical (37).

3. MATERIALS AND METHODS

3.1. Materials

Amino acids and resin for the peptide synthesis were supplied by CEM. All other chemicals were purchased from Sigma Aldrich unless otherwise stated. Bacteria strains used during the research were *Escherichia coli* (ATCC 25922 NTCC 13846), *Staphylococcus aureus* (ATCC 29213, ATCC 25923), and *Methilicin- Resistant Staphylococcus aureus* (MRSA).

3.2. Thiolated Antimicrobial Peptides

3.2.1. Solid phase peptide synthesis (SPPS) of cysteine (Cys) ended antimicrobial peptides (C-P1 and P1-C)

C-AMP (C-P1 and P1-C) were synthesized with The Liberty Blue™ Automated Microwave Peptide Synthesizer (CEM). The synthesis scale was 0,1 mmole and 0,143 mg Rink Amide Protide resin was previously weighed, left to swell in dimethylformamide (DMF) for at least 30 minutes prior to peptide synthesis. The conformation of the amino acids in the sequence of C-P1 and P1-C were Arginine (Arg, R) and Cysteine (Cys, C) in L form, Leucine (Leu, L) in D form. 4,68 grams of R in L form was weighed and dissolved in 36 mL of DMF. Similarly, 2,2 g of L in D form and 0,36 g of C in L form were weighed and dissolved in DMF.

Table 3.1. Sequential information about synthesized C-AMPs

Cysteine Added Antimicrobial Peptides (C-AMP)	Amino Acid conformation	pI	Net charge	Molecular weight (g/mol)
C-P1 (CRlllRllRRllRlllR)	C – L form R – L form	13.2	+7	2188.89
P1-C (RlllRllRRllRlllRC)	L – D form	13.2	+7	2188.89

N,N'-Diisopropylcarbodiimide (DIC) was used as activator and 2,2 mL of DIC was completed to 28 mL with DMF. For activator base oxyma was used and 1.0 M oxyma was prepared in 14 mL of DMF. Deprotection cocktail was prepared with 20% Piperdine (v/v) in DMF.

At the end of the peptide synthesize, peptide was cleaved from the resin with Razor^{TM} Peptide Cleavage System (CEM). The cleavage cocktail consists of 4.75 mL of trifluoroacetic acid (TFA), 125 µL of triisopropylsilane (TIS) and 125 µL of distilled water (dH_2O). The peptide cleavage was carried out at 38°C for 45 minutes.

The cleaved peptide was precipitated in cold diethyl ether and then centrifuged 4000 rpm for 3 minutes. After centrifugation diethyl ether was carefully discarded and peptide pellet was left to dry.

3.2.2. Purification and characterization of C-AMPs

Synthesized C-AMPs (C-P1 and P1-C) were prepared in 50% acetonitrile (ACN) to have a concentration 2,5 mg/mL and sonicated to dissolve. Dissolved C-AMP were purified by using 1260 Infinity Quaternary LC (Agilent Technologies) systems with

prep RP-HPLC hydrophobic 6 µm C18 column (Agilent VariTide RPC 250x10 mm ID). The column equilibrated by 5% mobile phase (B solution was ACN and A solution was dH_2O containing 0,025% TFA). Synthesized peptide samples were first run in a linear gradient in between 1-80% B% for 30 minutes to scan at which % the peptide peak can be seen. After detecting the B% of the sample peak, between 55-70% B was focused. Relevant pure peaks for C-AMPs were collected with fraction collection. The excess ACN was evaporated with Rota Evaporator, then lyophilized with Freeze Dryer (Labconco).

Purified C-AMPs were characterized with 6420 Triple Quad LC/MS (Agilet Technologies) system. C-AMPs were prepared dissolving 1 mg in 1 mL of 50% ACN. Initially, 10 µL of samples were directly injected to mass detector to be analyzed in scan mod between 50 to 2200 mass (m) range for 1 minute. Mobile phases contain 0.05% formic acid (FA) and samples were injected to mass detector in the 50% ACN. After scanning, samples were analyzed in single ion monitoring (SIM) mode for the 3 distinct charge (z) ions that were seen in the scan spectrum. The 3 SIM modes were for 2188.89 m/z, 312.7 m/z, and 548 m/z ions.

3.2.3. Concentration detection of C-AMPs

Concentration of the C-ended AMPs by synthesized SPPS method was done with the Pierce™ Quantitative Fluorometric Peptide Assay (Thermo Scientific™). The final concentration of the standard A was 841 µM for individual peptide concentration measurement and used as stock standard solution. All standards were prepared according to the commercially obtained kit manual. 10 µL of each assay standard and C-AMP samples were pipette into 96-well black plate. 70 µL of Fluorometric Peptide Assay Buffer was added on top of the assay standards and C-AMP samples. 20 µL of Fluorometric Peptide Assay Reagent were added into each well. Samples and the standards were incubated at room temperature for 5 minutes in dark. Fluorometric

measurement were done at Ex/Em 390 nm/475 nm. To convert the concentration of the C-AMP samples from µM to µg/mL the following equation was used;

$$\text{Concentration } (\mu g/mL) = \text{ Mwt of Peptide (Da)} \times \text{ Concentration } (\mu M)/10^6 \quad \text{(eq.1)}$$

3.2.4. Quantitating sulfhydryl groups with Ellman's reagent assay

The concentration of the sulfhydryl group due to the C amino acid in the sequence of the AMP was detected with Ellman's Reagent (Thermo Scientific^{TM}). Prior to sulfhydryl group quantitation the known concentration of C-P1 and P1-C were prepared in 50% ACN. For the preparation of standards L-Glutathione reduced (GSH) was used. The final concentration of the standard A was 1.5 mM prepared in reaction buffer (0.1 M sodium phosphate, 1 mM EDTA, pH 8.0). All standards were prepared according to the assay manual (38). Ellman's reagent solution was prepared by dissolving 4 mg of Ellman's reagent in 1mL of reaction buffer.

To control the possibility of forming a disulfide bond (S-S) in between the -SH groups in the C-AMPs upon oxidation, peptides were treated with a strong reducing agent dithiothreitol (DTT) (0.01% (w/v)) for 1.5 hour at room temperature. C-AMPs and DTT were prepared in 50% ACN. After 1.5 hour of DTT treatment, peptide solutions were analyzed firstly with HPLC with the same scanning gradient with C-AMPs and then collected sample peaks were analyzed with Ellman's assay.

A set of test tube were prepared containing 50 µL Ellman's Reagent and 2,5 mL of reaction buffer. 250 µL of prepared known concentration of C-AMPs and each assay standards were pipette into the test tubes according to the label. Sample solutions in the test tubes were mixed and incubated at room temperature for 15 minutes. At the

end spectrophotometric measurement of all standards and samples were performed at 412 nm.

By using the absorbance values measured at 412 nm the -SH amount was calculated by using the Beer-Lamber law. The concentration values were calculated with standard curve and the molar absorptivity coefficient was taken as 14.150 $M^{-1}.cm^{-1}$ which is the molar absorptivity coefficient value of 2-nitro-5-benzoic acid (TNB). The calculated values were multiplied with the dilution factor 0.0028 which was the total measured volume (2.8 mL). After all calculations were done the results of DTT treated untreated C-AMPs were compared.

3.2.5. Minimal inhibition concentration (MIC) of C-AMP

E.coli 13846, *E.coli* 25922, *S.aureus*29213, *S.aureus* 25923, and MRSA, were incubated in Mueller-Hilton Broth (MHB) overnight at 37°C. The OD600 measurement that was done with Gen5 Micro plate reader, of the overnight bacteria incubations which were 0,5 McFarland ($1.0x10^8$ cfu/mL) adjusted to 0,1. OD600 adjusted bacteria cultures were diluted 1:100 ratio with MHB. Serial dilutions of C-AMPs were prepared in MHB in 96-well plate and 5 µL of bacterial suspension added into each well that contained different dilutions of C-AMPs. At the end the highest concentration of the AMPs was 128 µg/mL. For controls Ampicillin and the non-thiolated version of the C-AMPs which is P1, were used as positive control. All bacteria plates were incubated 18-20 hours at 37°C and all the C-AMPs treatments were carried triplicate. The image of the plates was taken with Bio-Rad ChemiDoc^{TM}MP Imaging System.

3.2.6. Hemolytic assay

Sterile Tris-Saline (10 mM Tris, 150 mM NaCl pH 7.2) solution was prepared for the hemolytic assay. 30 µL of fresh blood and 10 mL of Tris-Saline solution mixed well together then centrifuged at 1500 rpm for 5 minutes. The supernatant was discarded, and the pellet was dissolved with 10 mL of Tris-Saline solution then centrifugation step was repeated. The red blood cell (RBC) pellet was dissolved with 10 mL of Tris-Saline solution and 100 µL added into each well of 96-well plate. On top of RBCs 100 µL of serial diluted C-AMPs were added. As a complete lysis control Triton X-100 was used. The plate was incubated at 37°C for 30 minutes. After incubation the plate was centrifuged at 1500 rpm for 10 minutes. The supernatants were transferred into a new 96-well plate and measured at 414 nm. The %lysis was calculated by using the following formula;

$$\%\text{lysis} = \frac{[\text{OD414 (Sample)} - \text{OD414 (Blank)}]}{[\text{OD414(Total Lysis)} - \text{OD414(Blank)}]} \text{x } 100 \quad \text{(eq.2)}$$

3.2.7. *In vitro* cytotoxicity assay

Mouse fibroblast 3T3 (ATCC CRL-1658), human epidermal keratinocyte HaCat (ATCC PCS-200-011) and human cervix epithelial adenocarcinoma HeLa (ATCC CCL-2) cell lines were incubated with Dulbecco's modified Eagle media (DMEM) which was supplemented with 10% fetal bovine serum (FBS) and 1% antibiotics (100 µg/mL of streptomycin and 100 U/mL of penicillin) at following conditions; 37°C, 5% CO_2, and 80-90% relative humidity. Approximately at 85% confluency the media was discarded, cells were washed with 1X phosphate buffer saline (PBS), trypsinized with 0.25% Trypsin-EDTA, and incubated at 37^{o}C for 5 minutes. Trypsin deactivated with DMEM, the detached cells were collected and centrifuged at 500 g for 5 minutes. The

supernatant was discarded, and the cell pellet was resuspended with DMEM. The resuspended cells were counted with hemocytometer.

For MTT cytotoxity assay (Roche), $5x10^4$ cells/well in 100 µL were seeded in 96-well plate. The 96-well plate was incubated at 37ºC, 5% CO_2 for 24 hours for cell attachment. After 24 hours of cell attachment, serial dilutions of C-AMPs, were prepared and 10 µL added to each appropriate well. At the end the concentration of the C-AMPs in wells started from 64 µg/ml. Magainin II (Mag2), a known antimicrobial peptide that has a non-toxic nature and the non-thiolated version of the C-AMPs which is P1, were used at the same concentrations as a positive control of the C-AMPs. All measurements were performed in triplicate. C-AMPs applied plates were incubated 18 to 24 hours at 37ºC, 5% CO_2. After incubation, to measure the toxicity effect of the C-AMPs, MTT protocol was applied. In each well 10 µL of MTT labelling reagent was added and then plates were incubated 37ºC, 5% CO_2 for 4 hours. After 4 hours of incubation, in each well 100 µL of solubilization solution (10% SDS in 0.01 M HCl) was added. The plates were incubated (37°C, 5% CO_2) overnight. After overnight incubation, the purple formazan crystals were solubilized, and the absorbance measurement of the samples were done at 550 nm and 690 nm with Thermo Scientific Varioskan Flash plate reader.

3.3. Silicone Catheter Surface Modifications

3.3.1. Cleaning and activating the surface of medical silicone catheter

Medical silicone catheter (RüschBrillant) was cut into approximately 0,5 mm pieces for the cleaning and activation procedure. The cleaning procedure was applied according to the ProChima Silane Surfaces protocol (39). For cleaning step, piranha solution, as an acidic solution, was prepared by mixing equal volumes of hydrogen peroxide (H_2O_2) and sulfuric acid (H_2SO_4). Prepared silicone catheter pieces were

treated with piranha solution for 20 minutes, then washed with distilled water. Piranha treated catheter pieces were then dried for 1 hour at 70°C under vacuum. Dried catheter pieces were stored under nitrogen gas for further usage.

3.3.2. Hydroxylation of silicone catheter surface by UV/ozone treatment

Hydroxylation of catheter surfaces were done using ProCleaner^{TM} unit (BioforceNanosciences). ProCleaner^{TM} unit was placed inside the fume hood to minimize the ozone leakage. Catheter pieces ready for surface modification placed on the pedestal of the ProCleaner^{TM} unit. Distance from the pedestal to the UV lamp was 0,3125 inches and the UV intensity was 19,39 mW/cm^2. The expose of UV-Ozone was set for 2 hours. To capture the produced radicals due to UV-Ozone treatment, 2 mM reduced L-Glutathione (GSH) solution was applied to silicone catheter pieces for 30 minutes at 25°C.

To control whether after the GSH treatment there were radicals left on the modified silicone catheter surface; triethylene glycol dimethacrylate (TEGDiMA) used as crosslinker and 2,2-dimethoxy-2-phenylacetophenone (DMPA) used as photoinitiator for the gelation control. TEGDiMA and 1% (w/v) DMPA mixture was used as a positive control. On the hydroxylated catheter surfaces both TEGDiMA and TEGDiMA-1% (w/v) DMPA mixture were dropped on the GSH treated silicone catheter surface and cured under 365 nm UV light for 1 hour. At the end of the curing the results were observed and recorded.

3.3.3. Attachment of intermediate molecule (allytrimethoxysilane)

5% Allytrimethoxysilane (ATS), 5% dH_2O and 90% acetonitrile (ACN) of intermediate molecule mixture was prepared and mixed thoroughly. Then previously

prepared silicone catheter pieces were treated with ATS mixture for 4 hours on magnetic stirrer at room temperature. After 4 hours of intermediate molecule ATS treatment, silicone catheter pieces were removed and washed with 100% ACN and acetone respectively. The washed silicone catheter pieces were dried on air for further C-AMPs immobilization.

3.3.4. Conjugation of C-AMPs to the modified catheter surface

The nucleophilic addition: 1 mg/mL C-AMPs solutions prepared in 50% ACN. 6,4 µL triethylamine (Et_3N) which is molar equivalent of C-AMPs, was added into the C-AMPs solution. Short intermediate molecule ATS bounded catheter surfaces were placed into the C-AMPS immobilization solution carefully without damaging the modified outer surface of the silicone catheters. C-AMPs binding reaction left to overnight immobilization on magnetic stirrer at room temperature. After overnight reaction, C-AMP immobilized catheter pieces were removed and washed with 100% ACN and acetone thoroughly. The washed catheter pieces were left to dry on paraffin film.

The free-radical addition: Similar to nucleophilic addition, 1 mg/mL C-AMPs were weighed and dissolved in 50% ACN via sonication. After preparing the C-AMP solutions, ATS bonded silicone catheter pieces mixed. 10 µL 0.02 mg of DMPA which 0.1 molar equivalent of C-AMP was added to the mixture. Samples were mixed under UV lamb (365 nm) for 1 hours with magnetic stirrer. After 1 hour of reaction, the peptide bounded catheter pieces were removed from the C-AMP solution and washed with 100% ACN and acetone respectively. The washed catheter pieces were left to dry on a paraffin film for further experiments.

3.4. Surface Characterization of C-AMPs Bonded Silicone Catheter Surface

3.4.1. Chemical characterization

The surface chemistry of C-AMPs immobilized catheter surface was measured with the Thermo^{TM} Nicolet iS10 FT-IR (Thermo Fisher) to determine the presence of the amide bonds. C-AMPs immobilized silicone catheter surfaces were placed as the modified face get into contact with the attenuated total reflection (ATR) sampling accessory. Each sample spectrum was recorded from 400 cm^{-1} to 4000 cm^{-1} with 32 scans at room temperature.

3.4.2. Physical characterization

Wettability of modified catheter surfaces were analyzed by contact angle measurement technique. The static water contact angle (CA°) and the surface hydrophobicity measurements of the C-AMPs immobilized, and unbounded silicone PDMS surfaces were performed with Attention Theta Lite Contact Angle (Biolin Scientific). 0.5 µL of water was dropped on the modified and unmodified silicone PDMS surface. The images of the dropped water droplet were taken with high magnification camera and the measurements were analyzed with the OneAttension software.

3.4.3. Morphological characterization

The semi- quantitative elemental analysis was done with scanning electron microscopy (SEM) (Thermo Quattro S) coupled with energy dispersive x-ray spectroscopy (EDS) (EDAX). C-AMP immobilized catheter pieces were coated with

20 nm of gold (Au/Pb) under vacuum. After Au coating, the semi-quantitative elemental analysis of the peptide immobilized catheter samples was done under high vacuum. To verify the immobilization, the elemental ratios of carbon (C), oxygen (O), nitrogen(N) and sulfur (S) atoms were recorded.

3.5. C-AMP Immobilized Silicone Catheter

3.5.1. Antimicrobial activity determination

Colony counting: Similar to MIC assay explained in section 3.2.5 *E.coli* 25922 and *S.auresus* 25923 were cultivated overnight in MHB at 37°C. OD600 of the bacteria adjusted to 0,1 which is equivalent to $1.0x10^8$ cfu. Bacteria inoculum was diluted to $1.0x10^5$ cfu/mL with MHB. C-AMP immobilized catheter pieces were placed in 40 µL of bacteria suspension as the coated surface would be in the contact. Uncoated silicone catheter was used as a control. The inoculum contacted C-AMP coated and uncoated catheters were incubated 40 minutes at 37°C. After 40 minutes of incubation, 10 µL of the inoculum was withdrawn and 8 serial dilution was done in the ratio 0f 1:10 with MHB. 10 µL of serially diluted inoculum samples were dropped on a MH agar and incubated overnight at 37°C for the cfu determination.

4. RESULTS

4.1. C-AMPs

4.1.1. Purification and characterization

After the synthesis of the C-AMPs, peptides were initially run with general gradient for 30 minutes to scan for impurities and detect the polarity. For C-P1, there were two peaks that observed at the polarity around 60-70% at 214 nm. Their purity was 59% and 14% respectively (Figure 4.1). It was suspected that these two peaks are belong to C-P1 sample. To be ensure for this the focused method was applied.

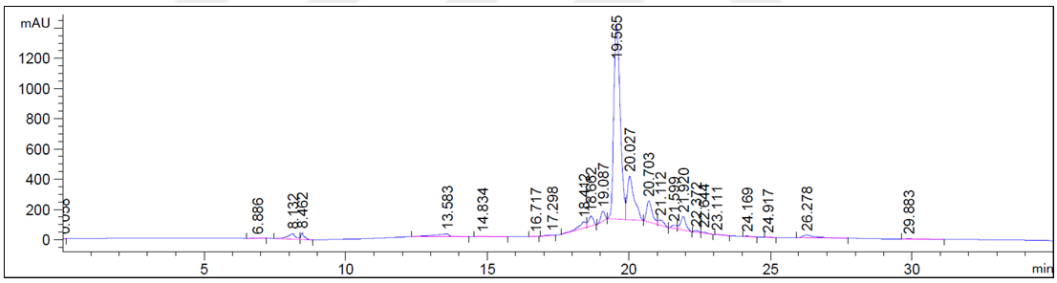

Figure 4.1. C-P1 RP-HPLC chromatogram with scan method.

Depending on the polarity when the 58-70% mobile phase B was focused there were one peak, and its purity was 97% (Figure 4.2).

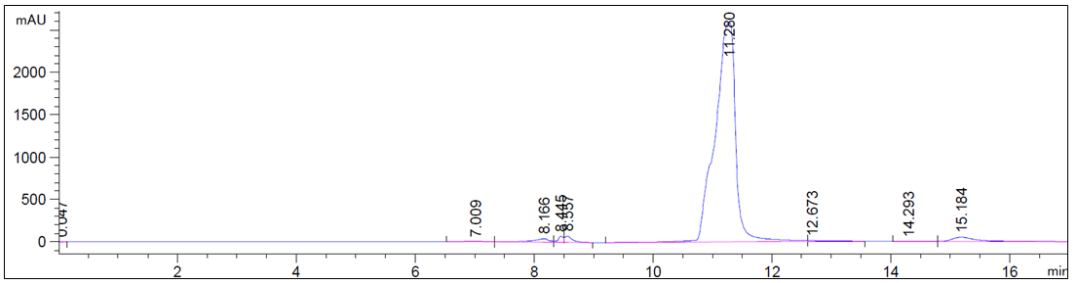

Figure 4.2. C-P1 HPLC chromatogram with focused method.

To control whether the -SH groups formed a S-S bond or not 1.5 hours of 0.1 % (w/v) DTT treatment was applied to C-AMPs. Later DTT treat C-AMPs were analyzed with HPLC with the same scanning method. Results were given below.

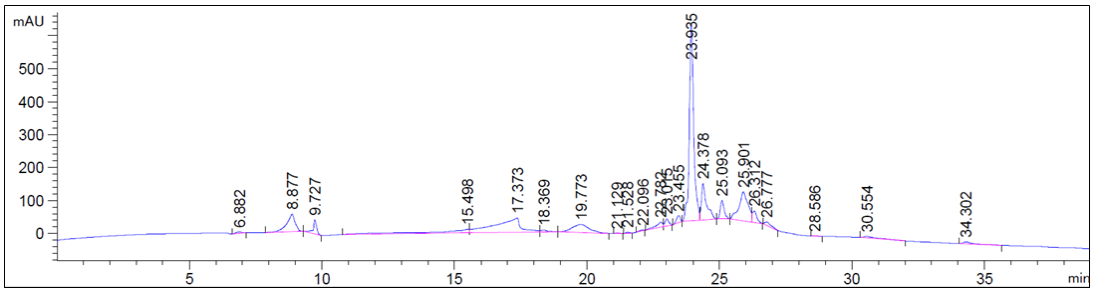

Figure 4.3. RC-HPLC analysis of DTT treated C-P1 in scan mode.

In the DTT treat C-P1, similar chromatogram (Figure 4.3) with the untreated C-P1 (Figure 4.1) was obtained. When looking at the HPLC chromatogram there is a minimal peak at the 8 minute which belong to the DTT. The similar two peaks obtained at the similar B% which indicates the same sample peaks.

Similar to C-P1, P1-C was also initially analyzed with RP-HPLC in general gradient to scan the impurities and determine the sample polarity at 214 nm. In P1-C chromatogram two peaks were observed around 60-70% mobile phase B as well which their purities were 45% and 26% respectively (Figure 4.4).

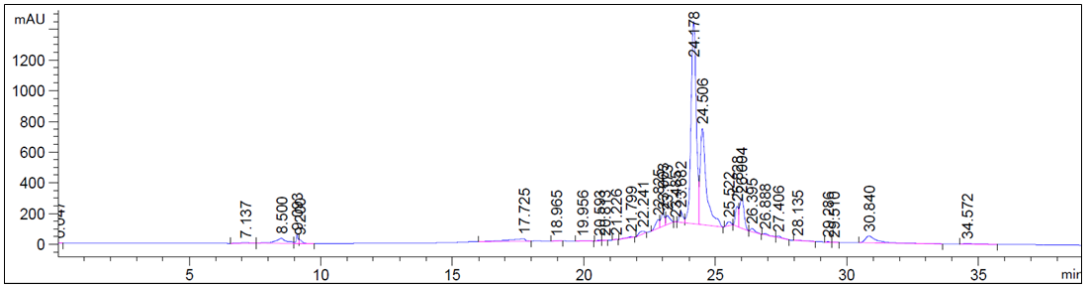

Figure 4.4. P1-C RP-HPLC chromatogram with scan method.

When polarity was focused in between 60-70% the peak number decreased to one and the purity was increased to 94% (Figure 4.5).

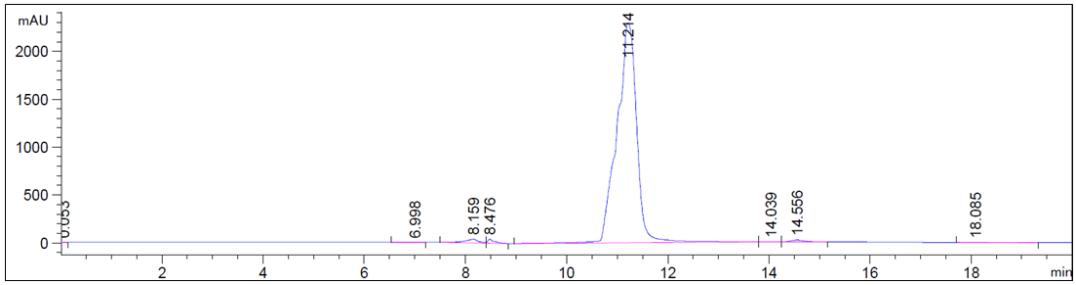

Figure 4.5. P1-C HPLC chromatogram with focused method.

The same DTT treatment and the HPLC analysis were applied to P1-C also in the same conditions. The HPLC chromatogram of DTT treated P1-C was given below in Figure 4.6. When looking at the chromatogram there is a peak at minute 8 which is observed in DTT treated C-P1 chromatogram at the same minute. This means that peak at minute 8 belongs to DTT. There are also still two peaks observed in DTT-P1-C (Figure 4.6) which is also observed in the P1-C chromatogram around the same minute meaning that these two peaks belong to the sample peaks.

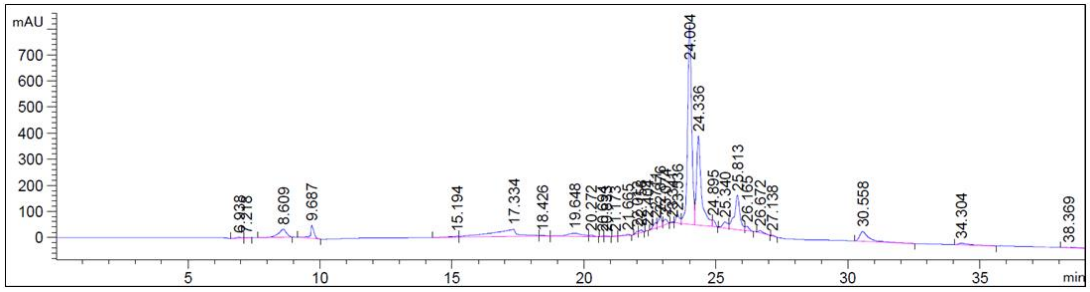

Figure 4.6. RC-HPLC analysis of DTT treated P1-C in scan mode.

In the LC-MS/MS it is expected to see 7 fragments of the C-AMPs due to the net +7 charge. Samples were analyzed in scan mode for 1 minute and the obtained scan spectrum and the spectrum of the fragments which are given below. The m/z values for both C-P1 and P1-C the same since their molecular weight and net charge are the same and they are listed in the Table 4.1.

Table 4.1. Net charge and corresponding m/z values for C-AMPs

Mass (s)	Charge (z)	Mass/Charge (m/z)
2188.89	+1	2189.89
	+2	1095.45
	+3	730.63
	+4	547.97
	+5	438.58
	+6	365.82
	+7	313.70

When looking at the scan spectrum (Figure 4.7.A) samples peak has a good abundance around 10^7. Samples were run in both negative and positive scan mode at which the fragments were majorly seen in positive scan mode (Figure 4.7.B). When the m/z values are compared with the positive scan mode the 5 peaks that are belong to fragments can be seen which are the +2, +3, +4, +5 and +6 charged fragments.

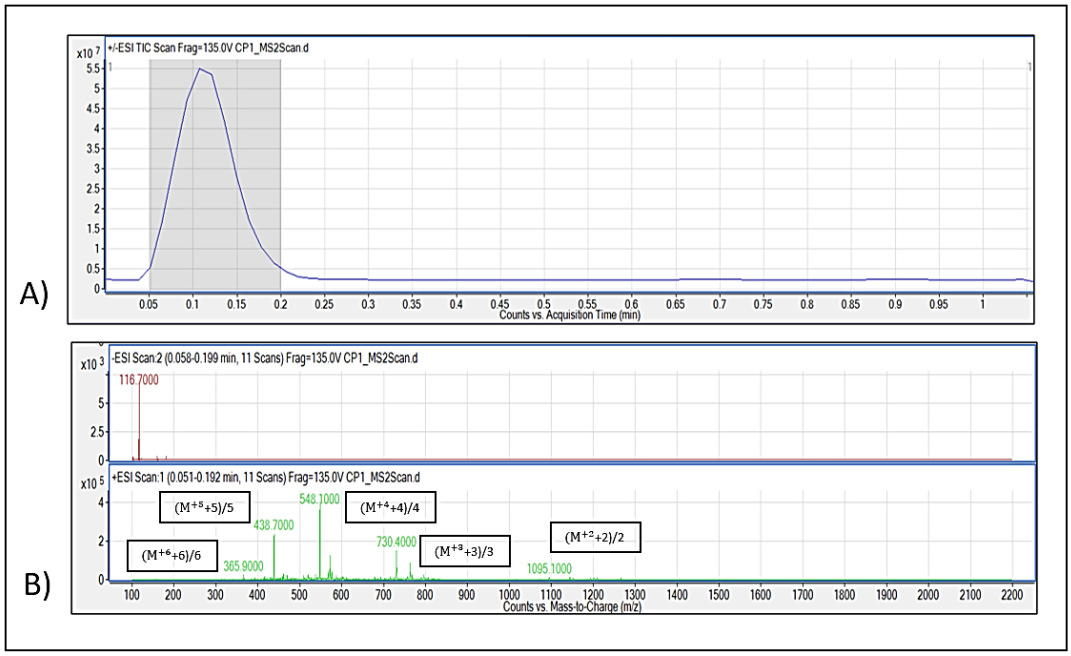

Figure 4.7. LC-MS/MS results of C-P1.
A) Sample peak in scan mode. B) Fragment peaks in negative and positive scan modes.

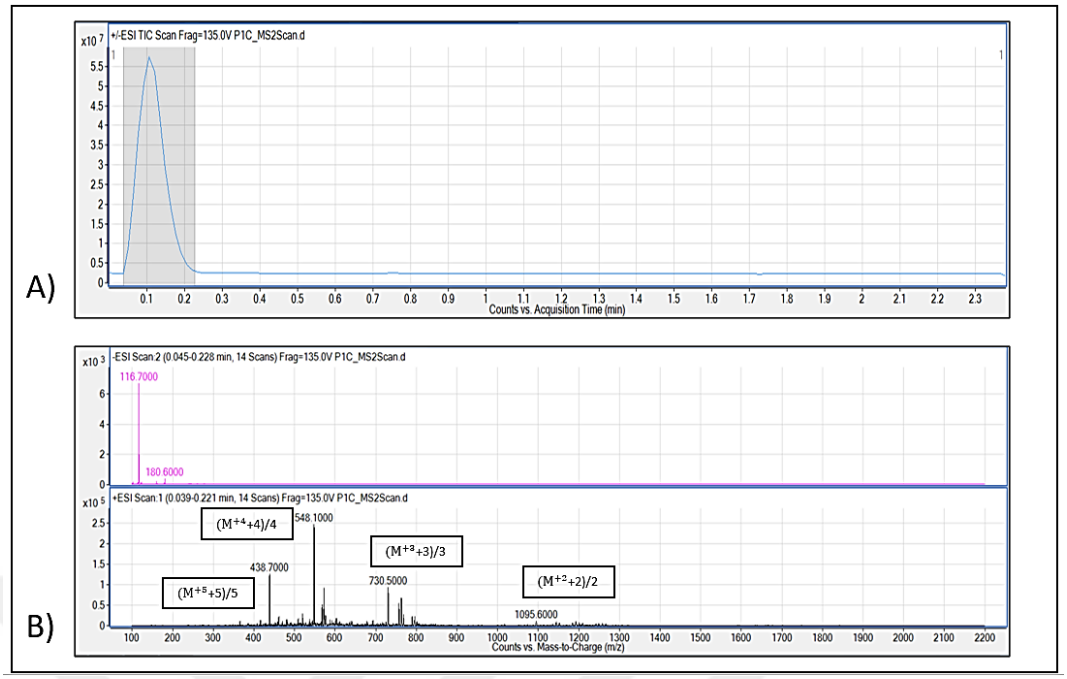

Figure 4.8. LC-MS/MS results of P1-C.
A) Sample peak in scan mode. B) Fragment peaks in negative and positive scan mode.

Similar to LC-MS/MS results of the C-P1, abundance of the sample peak of P1-C also measured around 10^7 (Figure 4.8.A). As detected fragments in positive scan mode, there were 4 detected fragments in Figure 4.8.B which are the +2, +3, +4 and +5 charged ones.

4.1.2. Concentration and sulfhydryl group determination

After purification and lyophilization, the concentration of the dissolved C-AMPs was measured with peptide concentration quantification kit. The lyophilized C-AMPs were dissolved in dH_2O. The molar concentration of C-AMPs was calculated with the standard curve. With the formula given in the method section (3.2.3) molar concentrations were converted into mg/mL. Further experiments were done by calculating the required peptide amount from the concentrations given in the table below (Table 4.2).

Table 4.2. Concentration comparison results of C-AMPs.

C-AMPs	Molar Concentration (µM)	Concentration (mg/mL)
P1-C	923.3	2.02
C-P1	1501.2	3.29

To control whether the -SH groups in the C-AMPs were free or they were form disulfide bond (S-S) Ellman's assay was applied. The theoretical amount of -SH that should be detected was 0.457 mM. When the 0.01% (w/v) DTT treated and untreated 1 mg/mL C-AMP samples were analyzed and -SH amount was calculated (Table 4.3).

Table 4.3. Calculated sulfhydryl amounts.

C-AMPs	-SH Amount (mM)
P1-C	0.317
DTT_P1-C	0.115
C-P1	0.343
DTT_C-P1	0.070

When the -SH amounts were compared there was a decrease can be seen in the sample that were treated initially with 0.1% (w/v) DTT and then collected with HPLC. Meaning that there was no S-S bond present in synthesized C-AMPs at the beginning.

4.1.3. Minimal Inhibitory Concentration (MIC)

The MIC values of both thiloated and non-thiolated versions of the P1 peptide are given in the table below (Table 4.4). When looking at the MIC results non-thiolated P1 has better efficacy against the thiolated version (C-P1 and P1-C). However, since in this project C-P1 and P1-C were used for the surface immobilization their MIC comparison is more important.

Against *E.coli* ATCC 25922 C-P1 had a MIC values of 2 µg/mL while P1-C had 2 µg/mL. C-P1 was found to be more efficient than P1-C and ampicillin which has a MIC value of 8 µg/mL.

For all bacteria strains used in MIC value of P1-C found to be the same which is 8 µg/mL. MIC value of C-P1 found to be changes in between 1 µg/mL and 2 µg/mL. This means that C-P1 is 4 times more effective and P1-C against *E.coli* ATCC 25922, *S.aureus* ATCC 25923 and MRSA; and 8 times more effective against *E.coli* NCTC 13846 and *S.aureus* ATCC 29213.

Table 4.4. MICs of C-P1 and P1-C against different bacteria strains.

Bacterial Strain	**C-P1 (µg/mL)**	**P1-C (µg/mL)**	**P1 (µg/mL)**
***E.coli* (NCTC 13846)**	1	8	0.5
***E.coli* (ATCC 25922)**	2	8	0.5
***S.aureus* (ATCC 29213)**	1	8	0.5
***S.aureus* (ATCC 25923)**	2	8	0.5
MRSA	2	8	0.5

4.1.4. Hemolytic Activity

Hemolytic activity of the thiolated peptides were measured with freshly collected human red blood cells. For 100% lysis Triton- X-100 was used positive control and hemolysis values 0.25- 32 µg/mL concentrations of C-AMPs were given in the figure below (Figure 4.9). When looking at the results it can be seen that C-AMPs reach HC50 value around 16 µg/mL which MIC values of both C-AMPs given in Table 4.4 are lower than the HC50 value.

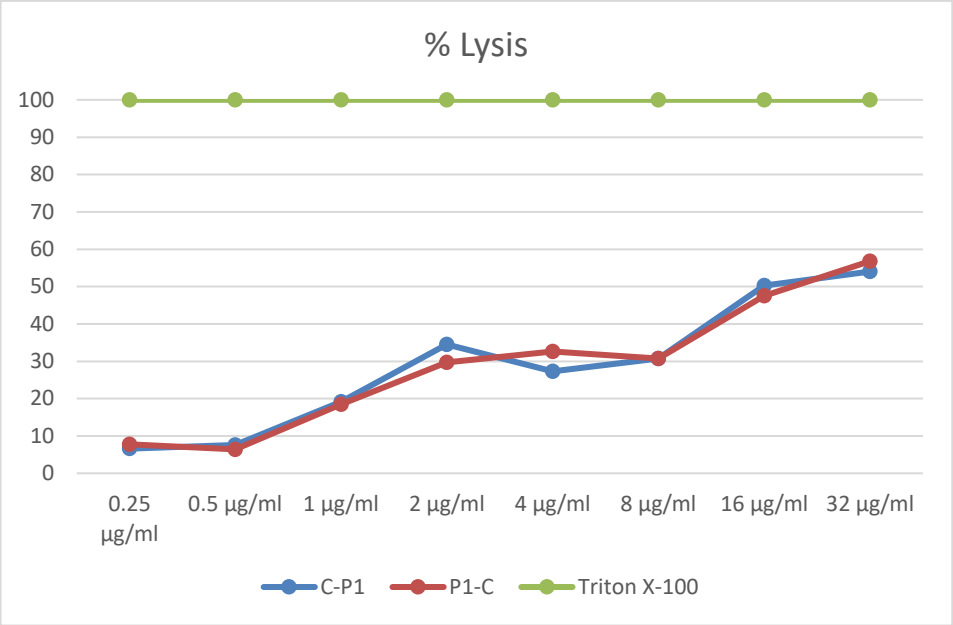

Figure 4.9. %Lysis results of synthesized peptides on fresh human red blood cells.

4.1.5. Cytotoxicity (MTT) results of C-AMPs

Toxicity effect of the C-AMPs were analyzed in three different cell types and for controls the non-thiolated P1 and Magainin-2 a known non-toxic AMP was used.

When looking at the cytotoxicity levels in mouse fibroblast cell line 3T3 (Figure 4.10), it can be seen that P1-C was less toxic at higher concentration comparing to C-P1, P1 and Mag-2. Until 1 µg/mL toxicity of P1-C and C-P1 are the same but after 1 µg/mL C-P1 becomes slightly more toxic than P1-C. C-P1 reaches the IC50 value after 4 µg/mL and P1-C passes IC50 after 16 µg/mL.

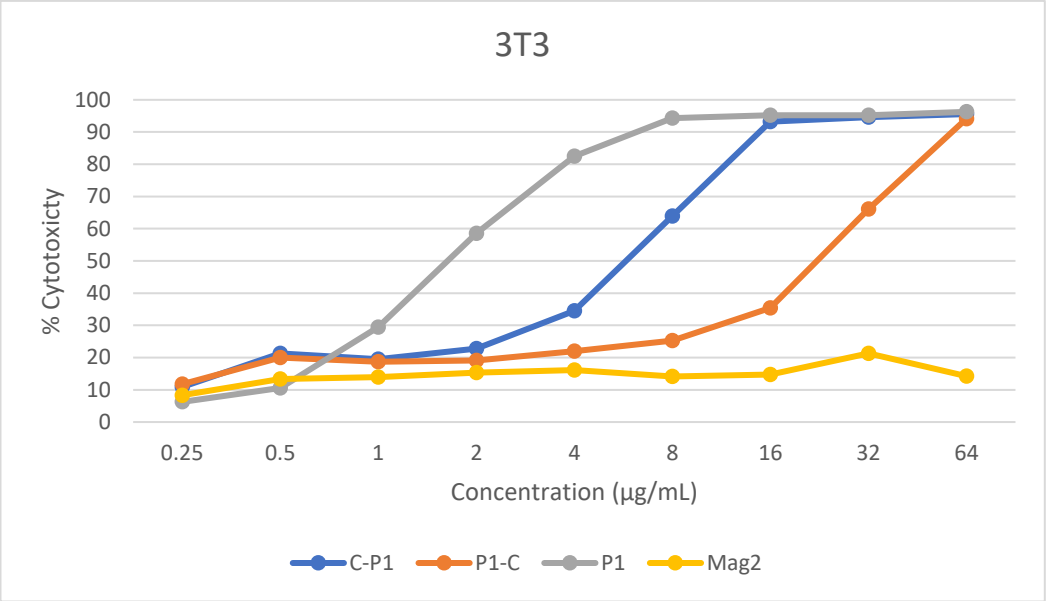

Figure 4.10. %Cytotoxicity results of synthesized peptides on 3T3 cell line.

In immortal human keratinocyte cell line (HaCat) at lower concentrations until 1µg/mL C-P1 was found to be less toxic than P1-C (Figure 4.11). The toxicity of the P1-C was slowly increase as the peptide concentration increases until 16 µg/mL, after 16 µg/mL the toxicity increased rapidly. At the concentration of 0.25 µg/mL, in between non-thiolated P1, C-P1, and P1-C, C-P1 found to be the least toxic.

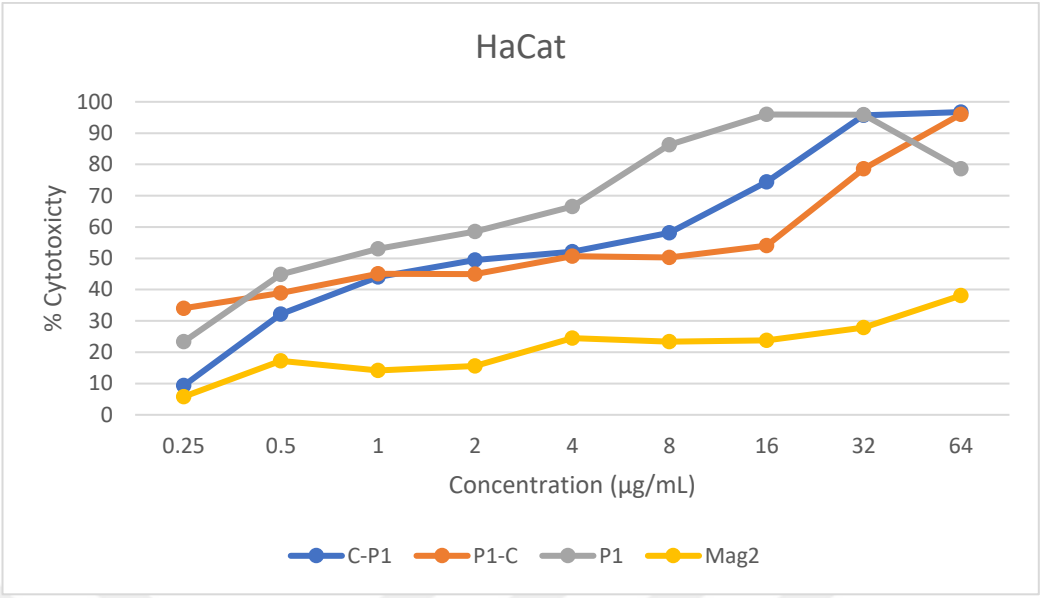

Figure 4.11. %Cytotoxicity results of synthesized peptides on HaCat cell line.

In Figure 4.12 the toxicity profiles of the non-thiolated P1 and C-AMPs were analyzed in HeLa cell line. The toxicity of P1-C is lower than 50% up to 16 µg/mL. At 32 µg/mL the toxicity level of P1-C increases to 80%.the toxicity of C-P1 increases slowly as the peptide concentration increases. C-P1 reaches 50% toxicity level after 4 µg/mL.

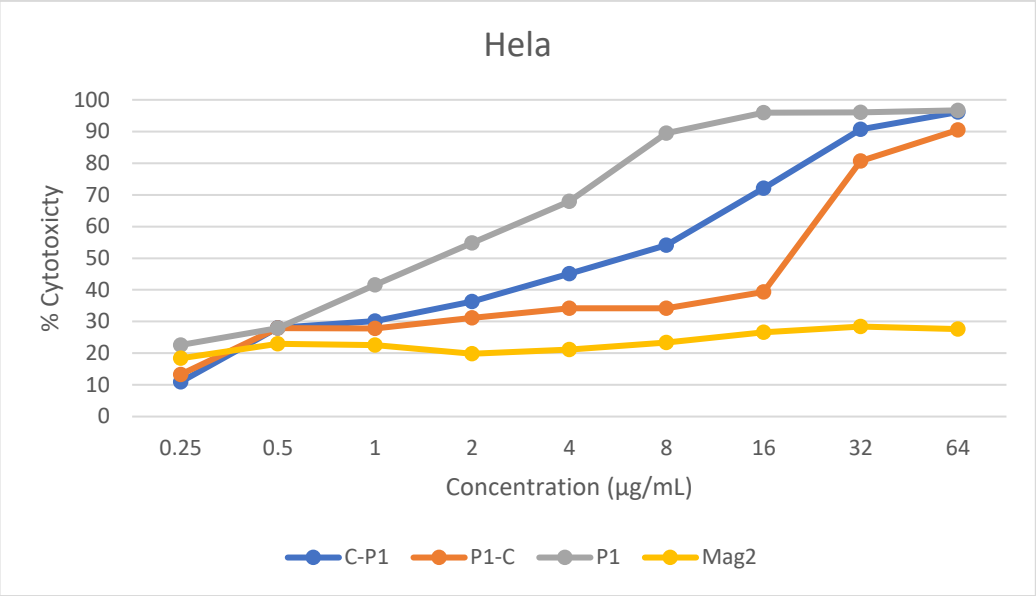

Figure 4.12. %Cytotoxicity results of synthesized peptides on Hela cell line.

4.1.6. Characterization of C-AMP Immobilization

4.1.6.1. Chemical characterization: FT-IR Spectroscopy

ATR/FT-IR spectrometry measurement was employed to verify the immobilization of for both free-radical addition and nucleophilic addition of C-AMPs on the medical silicone catheter surfaces. The ATR/FT-IR measurement was done with 32 scans in between 4000 to 400 cm^{-1}. The FT-IR spectrum of the untreated silicone catheter given in Figure 4.13 shows the characteristic peaks belongs to silicone, strong peak around 1074 belongs to the stretching vibration of the Si-O-Si bond. There are two peaks belongs to $Si-CH_3$, the one is around 1400 cm^{-1} due to the symmetric stretching caused by CH_3 and the other one is at 1258 cm^{-1} belong to $Si-CH_3$ stretching due the asymmetrical stretching of CH_3. Finally, around 2900 cm^{-1} there is a peak belongs to the C-H stretching.

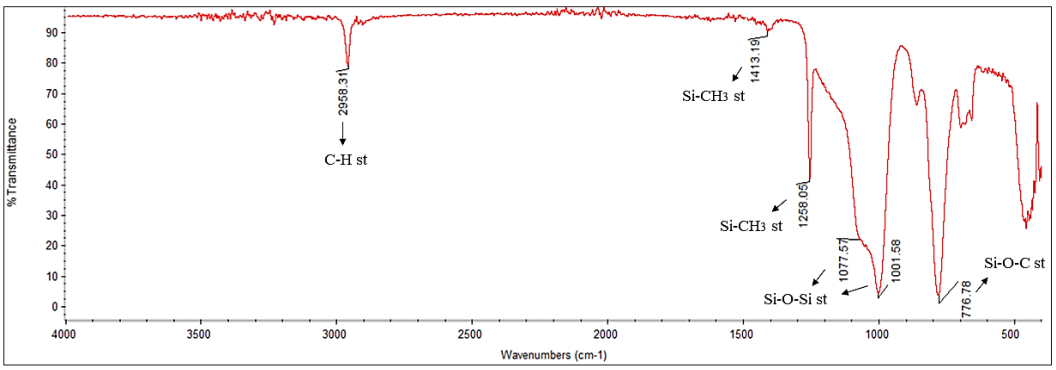

Figure 4.13. ATR-FT-IR spectrum of the untreated silicone catheter surface. (st=stretching).

The expected result initially the UV/O_3 treatment and later the 2 mM GSH treatment there should be a broad peak around 3500-3000 cm^{-1} that belongs to the hydroxyl groups (OH) which can be seen in the given figure below (Figure 4.14).

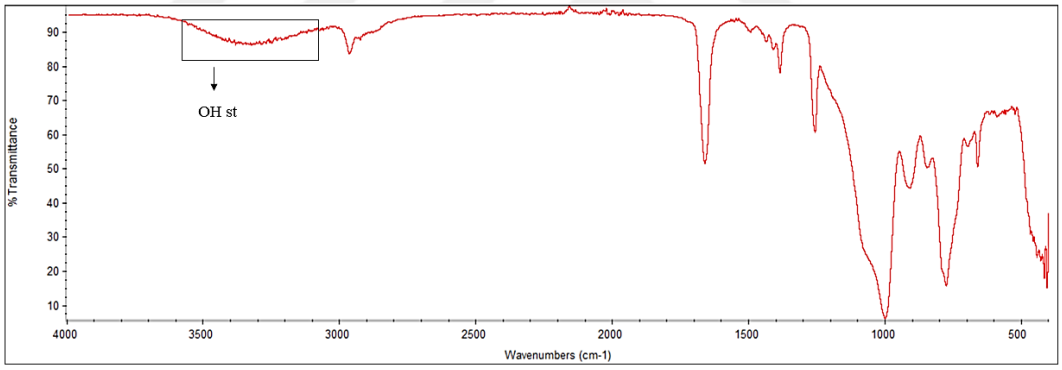

Figure 4.14. ATR/FT-IR spectrum of 2 hours UV/Ozon and 30 minutes 2 mM GSH treated silicone catheter. (st=stretching).

For peptides the bands that are looked in a FT-IR spectrum are the amide A, amide I and amide II bending peaks. Amide A located around 3500 cm^{-1} as a broad peak and represents the N-H stretching band. Amide I and II bending peaks are characteristically seen in the double bond region of the FT-IR spectrum which is around 1650 cm^{-1}. For both free-radical and nucleophilic immobilization methods the FT-IR spectrum was given below (Figure 4.15 and Figure 4.16). To compare, the spectrums of the sample peptides are analyzed together.

Carboxyl (C) terminus thiolated P1 peptide (P1-C) immobilized to silicone catheter surface with ATS intermediate molecule via two different TEC reaction. In Figure 4.15, the blue spectrum belongs to the nucleophilic P1-C addition and red spectrum belongs to the free-radical P1-C addition. When two spectrums are compared the expected amide A, I and II bands are clearer in the red spectrum (free-radical TEC reaction) of P1-C.

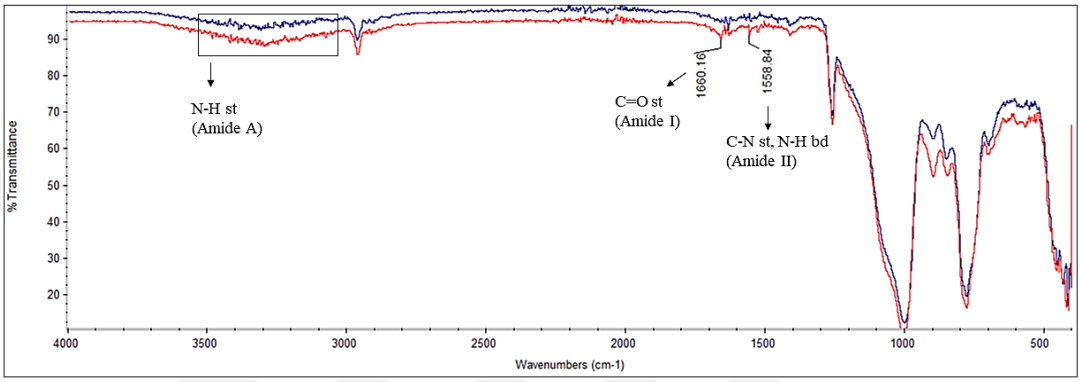

Figure 4.15. ATR/FT-IR spectrum comparison of two peptide conjugation method of P1-C. Blue spectrum is nucleophilic TEC, red free-radical TEC immobilization. (st=stretching, bd=bending).

Figure 4.16 represents the immobilization of the amide (N) terminus thiolated P1 peptide (C-P1). The green ATR/FT-IR spectrum belongs to nucleophilic addition of TEC reaction and red ATR/FT-IR spectrum belongs to the free-radical addition of TEC reaction. Similar to P1-C spectrums, Amide A, I and II stretching bands are more significant in the red spectrum which is the free-radical addition TEC reaction.

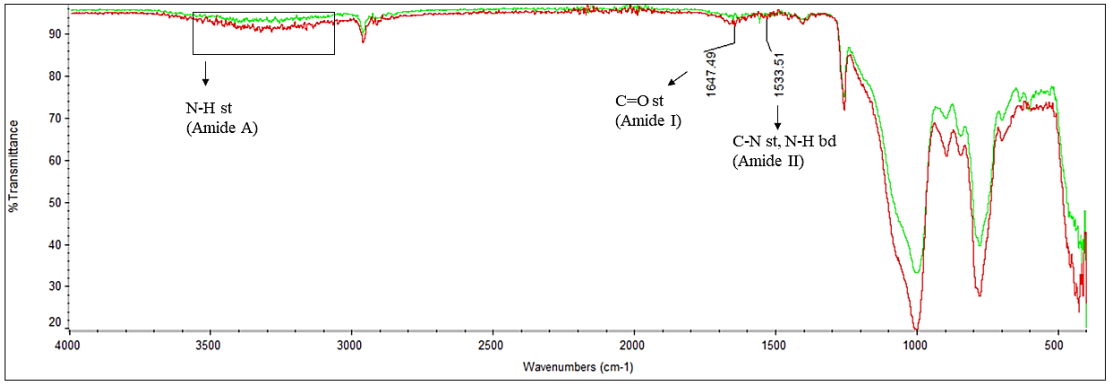

Figure 4.16. ATR/FT-IR spectrum comparison of two peptide conjugation method of C-P1. Green spectrum is nucleophilic TEC, red free-radical TEC immobilization. (st=stretching, bd=bending).

4.1.6.2. Physical characterization: Contact Angle Measurement

Surface hydrophobicity of untreated and C-AMP treated silicone PDMS surface was measured with contact angle of 0.5 µL dH_2O. The measurement the results were given in the table below (Table 4.5). The CA° of untreated silicone PDMS measured 105.25 meaning that the surface is hydrophobic. After the silicone surfaces immobilized with C-AMPs in both nucleophilic and free-radical addition method their CA° was measured. After the nucleophilic addition method CA° of the silicone PDMS surfaces are closer but higher than 90° meaning that the surface hydrophobicity was decreased but still hydrophobic. On the other hand, after immobilizing C-AMPs with free-radical addition method the surface CA° was dropped below 90° which means that the surfaces became hydrophilic.

Table 4.5. Contact angle measurements of distilled water on C-AMP treated and untreated silicone PDMS surfaces.

Sample	Contact Angle (CA°)
PDMS	105.25
C-P1$_{via}$Et$_3$N	98.74
P1-C$_{via}$Et$_3$N	93.98
C-P1$_{via}$DMPA	65.10
P1-C$_{via}$DMPA	73.89

4.1.6.3. SEM - Energy dispersion x-ray spectroscopy (EDS) analysis

Surface images and the semi-quantitative elemental analysis of the C-AMP immobilized catheters were done with SEM-EDS. For EDS analysis image of 100 µm area was taken and the elemental compositions were mapped. To compare the elemental composition of the C-AMPs in both nucleophilic addition (Et_3N) and free-radical addition (DMPA) methods, peptide mapping results were given in the table below (Table 4.6).

Table 4.6. Atomic percentage of elements obtained from EDS analysis for both TEC reaction of C-AMPs immobilization.

Element	C-P1$_{via}$Et$_3$N	C-P1$_{via}$DMPA	P1-C$_{via}$Et$_3$N	P1-C$_{via}$DMPA
C	19.12	26.98	19.06	26.93
N	3.41	2.60	2.96	2.80
O	76.71	68.67	77.04	68.26
S	0.76	1.75	0.95	2.01

4.1.7. Antimicrobial Activity of C-AMP Immobilized Catheters

4.1.7.1. Colony counting

For determining the antimicrobial activity, the coated surfaces of the C-AMPs immobilized catheters were submerged into the 40 µL of $1x10^5$ cfu/mL bacteria inoculum for 40 minutes. Then 10 µL of inoculum was serially diluted in the ratio of 1:10 for 8 times and 10 µL of each dilution were dropped on a MH agar. After overnight incubation the visible and countable colonies were counted. The images of the plates and the counts were given below.

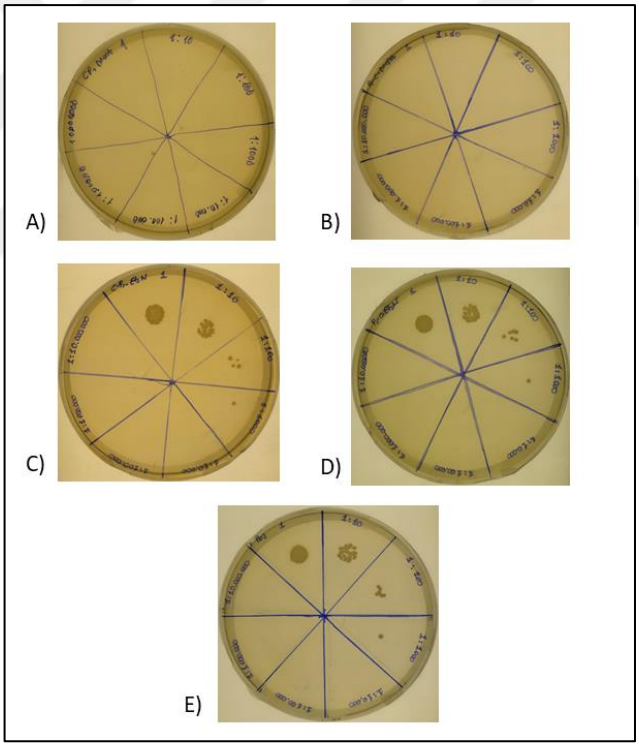

Figure 4.17. Antimicrobial activity of C-AMPs$_{via}$Et3N and C-AMPs$_{via}$DMPA against *E.coli* 25922. A) C-P1$_{via}$DMPA. B) P1-C$_{via}$DMPA. C)C-P1$_{via}$Et3N. D) P1-C$_{via}$Et3N. E) Untreated silicone catheter.

When looking at the Figure 4.17 different colony densities at different dilutions can be seen. There are no colonies that can be seen in the MH agar plates of the C-$P1_{via}$DMPA and P1-C_{via}DMPA. However, in the MH agars of untreated, C-$P1_{via}Et_3N$ and P1-$C_{via}Et_3N$ treated catheters there are colonies that were grown and can be counted. In all three agars (Figure 4.17.C, 4.17.D and 4.17.D) there are one colony at 10^{-3} dilution and 4, 6 and 7 colonies at 10^{-2} dilution respectively (Table 4.7). Meaning that free radical addition method for peptide immobilization is more effective than nucleophilic addition.

Table 4.7. Colony counts of C-AMP treated and untreated catheter surfaces against *E.coli* 25922.

	Untreated Catheter	**C-$P1_{via}$DMPA**	**P1-C_{via}DMPA**	**C-$P1_{via}Et_3N$**	**P1-$C_{via}Et_3N$**
Initial Bacterial Concentration (cfu/mL)	**Colony Count (cfu)**	**Colony Count (cfu)**	**Colony Count (cfu)**	**Colony Count (cfu)**	**Colony Count (cfu)**
$1x10^5$	7	0	0	4	6

5. DISCUSSION AND CONCLUSION

AMR has always been a serious problem that affects all living organisms include even plants and animals. There are various reasons that cause this problem such as benighted and excess usage of antibiotics both in agriculture and animal sectors as well as in medical treatments. Significant increase in the pollution in nature along with the population increase cause mutations in microorganisms. Accumulation of all these incidents results in the formation and the progression of the AMR. Development of new antibiotics is one of the solutions. Designing and production of new and effective antibiotics require significant time. However, the substantial acceleration in the progression of AMR hampers this process. Due to this, new solutions are investigated for AMR. One of the solutions for AMR is the AMPs.

AMPs can be naturally found in the immune system of the living organisms, or they can be designed synthetically. AMPs that are used in this thesis project are designed based on the amphipathic nature of the natural AMPs. Sequence of thiolated P1 AMP consist of 17 amino acids at which 6 AA residues are positively charged R in L-form, 10 amino acid residues are hydrophobic L in D-form, and 1 amino acid residue is C in L-form located at the N- or C-end of the peptide sequence. C-AMPs were synthesis with solid-phase peptide synthesis. In both sequence (C-P1 and P1-C) the ending was amide group which contributes overall the net positive charge of the peptides.

After synthesis process, C-AMPs were analyzed with LC-MS/MS for their chemical structure and molecular weight. C-AMPs have net +7 charge which 6 of them due to the 6 R residues in the peptide sequence and the remaining +1 charge is due to the amide end. In Table 4.1, the m/z ratios of various corresponding charges are listed. When looking at positive scan C-P1 LC-MS/MS in Figure 4.7, 5 out of 7 expected fragments which their m/z values are 365, 438, 730, and 1095 ($gmol^{-1}$) corresponding

to +2, +3, +4 and +5 charges can be detected. Similar situation for P1-C is observed in Figure 4.8. For P1-C positive scan, only +2 charged fragment that has m/z value 365 $gmol^{-1}$ did not be seen in the spectrum.

The purity of the synthesized peptides was analyzed with RT-HPLC. Figure 4.1 and Figure 4.4 demonstrate HPLC chromatogram of C-P1 and P1-C, respectively. These two peaks can be either due to the possible disulfide bond in between C-AMPs dimer or due to the different protonation amount caused by the TFA in the mobile phase. To control the disulfide bond formation possibility, C-AMPs were treated with DTT, which is a strong reducing agent reducing the sulfhydryl groups present in the C amino acid in the peptide sequences. Since DTT is reactive reducing agent, C-AMPs treated with DTT at room temperature for 1.5 hours. When DTT treated C-AMPs were analyzed with RT-HPLC, if there is a disulfide bond formed between C-AMPs the two peaks that were seen in the scan mode should become one peak. However, in both Figure 4.3 and Figure 4.6 the same two peaks were observed again. Also, there was a small peak that was suspected to belong to DTT was seen at the beginning of the run. To ensure that there was not disulfide bond formation after the cleavage of the peptides, before and after DTT treatment, Ellman's assay was conducted to measure the free sulfhydryl group amount which their results were given in the Table 4.3. Before DTT treatment the free sulfhydryl amount of C-P1 and P1-C were 0.343 mM and 0.317 mM respectively. After DTT treatment and HPLC analysis, Ellman's assay applied to the collected sample peaks and the calculated sulfhydryl amounts were for C-P1 was 0.070 mM and for P1-C was 0.115 mM. These results mean that were not any formed disulfide bond between C-AMPs. Which means that the initially detected two peaks in both C-P1 and P1-C HPLC chromatograms were due to the different amounts of protonated versions for the same peptide molecule.

The antibacterial activity of the C-AMPs was determined with MIC given in the Table 4.4. It can be seen that the thiolated versions of the P1 have higher MIC values yet still lower that ampicillin which was used as a positive control. Compared to C-P1

and P1-C together, C-P1 was found to be more effective against both gram positive bacteria *S.aureus* strains and gram negative *E.coli* strains.

Cytotoxicity effect of the C-AMPs were analyzed with MTT assay. In all three cell types non-thiolated P1 showed more toxic affected than the C-AMPs. When comparing the MIC values of C-P1 which is 1 µg/mL and 2 µg/mL with the MTT results it can be said that the effective concentrations against bacteria found be non-toxic for 3T3 and HeLa cell line. On the other hand, for HaCat cell line 2 µg/mL concentration was found to be at the 50% toxicity range. For P1-C the MIC values was 8 µg/mL which was found be to lower than 50% toxicity range in all three cell lines. Similar to MTT test results, the effective MIC values did not show a hemolytic affect against freshly collect human red blood cells which can be seen in the Figure 4.9.

While analyzing C-AMP immobilized catheter surfaces with ATR/FT-IR characteristic features of the peptides were looked for which are the amide vibrations that involve the following bonds of the peptide bond C=O, N-H, and C-H. These vibrations of the bonds are named and located at specific spectral regions. The three major bonds that are determined in the C-AMP coated silicone catheter surfaces are amide A, amide I and amide II (40). In this thesis two types of peptide immobilization methods (nucleophilic addition (C-AMPs$_{via}$Et$_3$N) and free-radical addition (C-AMPs$_{via}$DMPA)) were used and compared. In Figure 4.15 blue spectrum belongs to P1-C$_{via}$Et$_3$N and red spectrum belongs to P1-C$_{via}$DMPA. Although ATR/FT-IR analysis provides the presence or the absence of the specific functional groups of interest, in this thesis the presence of amide A, amide I, and amide II groups, it does not seem to provide the exact amount. However, depending on the size of the amide A, amide I, and amide II spectral band it can be said that more peptide molecules immobilized on to catheter surface, the spectral bands become larger. In Figure 4.15 especially the difference in the amide I stretching band can be seen meaning that more peptides were immobilized to catheter surface in P1-C$_{via}$DMPA method. Similar situation can be seen in Figure 4.16 which represents the ATR/FT-IR analysis of C-

P1. Green spectrum belongs to C-P1$_{via}$Et$_3$N and red spectrum belongs to C-P1$_{via}$DMPA. There is a significant difference in the amide A and amide I stretching bands of the C-P1$_{via}$DMPA. These ATR/FT-IR results means that for the C-AMPs immobilization to silicone surface, UV-assisted free-radical addition reaction is more efficient than nucleophilic addition reaction.

For the surface hydrophobicity determination contact angle (CA°) measurements could not be done with the peptide immobilized catheter pieces. Since catheter pieces are originally cylindrical shape, catheter pieces were cut in half in the dimensions of 0.5 cm x 0.5 cm to be able to immobilize the C-AMPs on the outer surface. However, their curve would not have the same angle, because the cutting process was done by hand not all the pieces would be the same. Although to eliminate this situation the volume of the droplet decreased to 0.5 µL, this causes a significant problem while measuring the CA° which would increase the standard deviation. On account of this, to be able see the effects of the C-AMPs immobilization on the surface hydrophobicity the CA° measurements was done with PDMS surfaces due to silicone based-structure of both PDMS and medical catheter. The same procedures were applied to flat PDMS surfaces and 0.5 µL drop of dH_2O was used. PDMS is a hydrophobic material which has a water CA° higher than 100 (41). The measured CA° values were listed in Table 4.5 and untreated PDMS surface gives a contact angle of 105. After the peptide immobilization the excepted CA° measurement was a decrement in the water angle even though the peptides were observed around 60% hydrophobicity in HPLC analysis. Although peptides could be count as hydrophobic, they are still more hydrophilic than untreated PDMS surfaces, so the CA° of C-AMP immobilized PDMS surfaces should lower than 100° and predicted to become more hydrophilic. Depending on this when the results were compared PDMS surfaces with treated with C-AMPs$_{via}$Et$_3$N measured higher than PDMS surfaces treated with C-AMPs$_{via}$DMPA. The difference in the angles could be due to the surface unevenness. Since PDMS molds were prepared by hand they were not perfectly flat which might affect the CA° measurement. Still, the obtained results were coherent with the ATR/FT-IR spectrums

which can be interpreted as more peptides can be immobilized to hydrophobic silicone surfaces via UV-assisted free-radical addition TEC reactions.

Semi-quantitative elemental analysis of the peptide immobilized silicone catheter surfaces was done with EDS detector assisted SEM technique. Measured elemental compositions were listed in the Table 4.6. The expected types of elements in the EDS mapping are carbon (C), oxygen (O), nitrogen (N), and especially sulfur (S) due to the sulfhydryl group at C amino acid residue. The reason for specifically S atom detection and its amount is important because it behaves like an indication of C-AMPs immobilization on to silicone catheter surfaces successfully. Until the EDS analysis it was seen that free-radical addition TEC reaction for C-AMP immobilization more effective. Depending on this information, when the atomic percentage of elements was compared the results were as anticipated; S atoms were detected almost 2 times more for the samples obtained via free-radical addition reaction compared to those modified by nucleophilic addition reaction.

After verifying all four atoms, C, O, N, and S, antimicrobial activity of the peptide immobilized catheter surfaces was investigated by treating the coated surface with certain colony of bacteria for colony counting. Coated surfaces were treated with 1x105 cfu/mL *E.coli,* ATCC 25922, for 40 minutes at 37°C. After bacteria treatment, inoculum was serially diluted at the ratio of 1:10 and 10 µL of this solution was dropped on MH agar plate. After overnight incubation, it was seen that for untreated catheter sample bacteria continue to grow and at 10^{-2} dilution 7 colonies can be counted. This was an expected result since there were not any substances that could obstruct the bacteria growth for this untreated catheter piece was used as a control. Considering the previous experiments, it was predicted that for the C-AMP immobilized catheter surfaces via free-radical addition TEC reaction, there should not be observed any countable colony. When looking at the Figure 4.17 the grown colonies on the MH agar plates belonging can be seen clearly in Figure 4.17.C, Figure 4.17.D, and Figure 4.17.E belonging to C-$P1_{via}Et_3N$, P1-$C_{via}Et_3N$ and untreated catheter pieces

respectively. There was not any countable colony formation in the Figure 4.17.A and Figure 4.17.B which were belonging to C-P1$_{via}$DMPA and P1-C$_{via}$DMPA. This was an expected result since all the surface characterization analysis free-radical addition method found to be more effective. With free-radical TEC reaction higher number of peptides can be immobilized to catheter surface and peptides can preserve their anti-bactericidal effect better.

To conclude, at the scope of this thesis, thiolated AMPs were designed by School of Medicine at ACU and the new and novel conjugation strategy was developed successfully at Nanobiotechnology Center at ACU. The effectiveness of the both C-AMPs alone and C-AMP immobilized silicone catheters was evaluated. Further experiments about the stability, antimicrobial and anti-biofilm activity of the peptide coated catheter surfaces are important to lead the way to test these new antimicrobial silicone catheters on living organisms.

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7. APPENDICES

Appendix 1. Peptide concentration determination

The peptide concentration measurements were done with Pierce™ Quantitative Fluorometric Peptide Assay by Thermo Scientific™. 8 standard dilutions were preapared with 1mg/mL peptide digest assay standard. Concentration measurements and calculations were done with the formula given as in 3.2.3. Measurements was done at Ex/Em 390/475 nm and the calibration curve is given below.

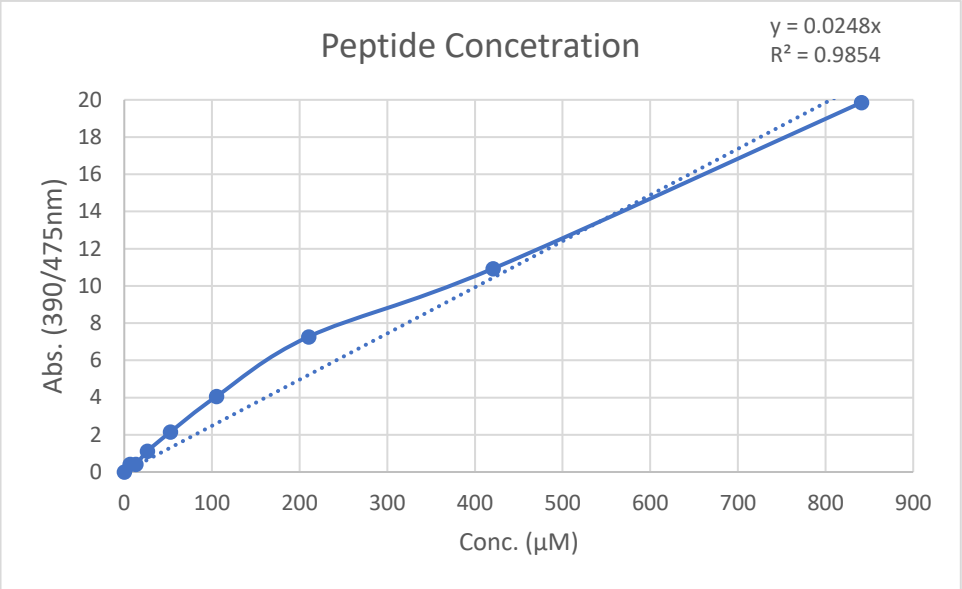

Figure 7.1. Standard curve for peptide concentration assay.

Appendix 2. The amount of sulfhydryl group in C-AMPs determination.

The amount of the free sulfhydryl (-SH) group present in the C-AMPs were determined with Ellman's Reagent by Thermo Scientific™.Reduced L-Glutathione was used as standard and 7 dilutes were prepared. Concentration measurements and calculations were done with the formula given in 3.2.4. Measurements were done at 412 nm and the calibration curve was given below.

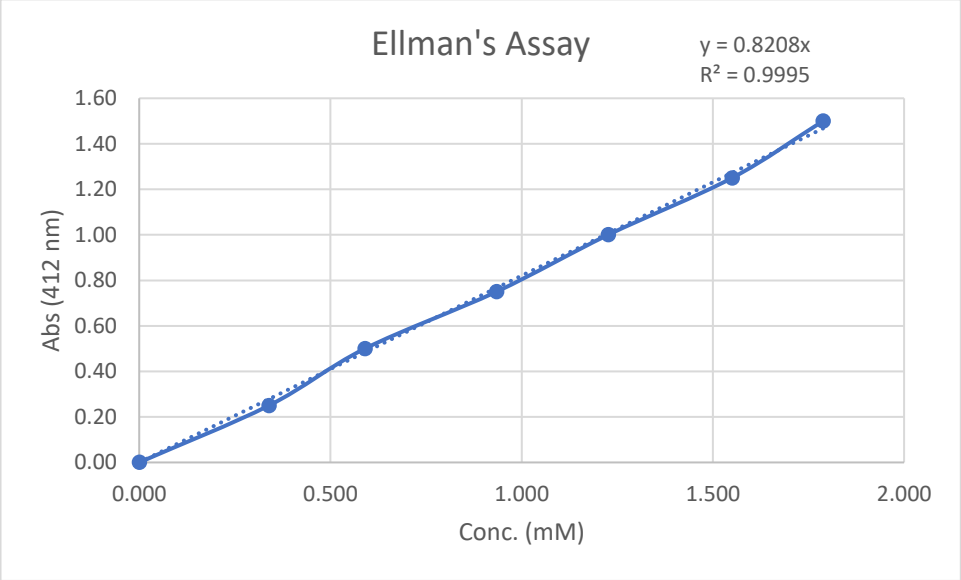

Figure 7.2. Standard curve for Ellman's assay.

8. CURRICULUM VITAE