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# SYNTHESIS OF PEPTIDE-FULLERENOL CONJUGATE AND PEPTIDE HYDROGELS FOR DRUG DELIVERY

A THESIS SUBMITTED TO
THE GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES
OF
MIDDLE EAST TECHNICAL UNIVERSITY

BY

HANDE DEMİRCİ

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR
THE DEGREE OF MASTER OF SCIENCE
IN
BIOMEDICAL ENGINEERING

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Approval of the thesis:

**SYNTHESIS OF PEPTIDE-FULLERENOL CONJUGATE AND PEPTIDE HYDROGELS FOR DRUG DELIVERY**

submitted by **HANDE DEMİRCİ** in partial fulfillment of the requirements for the degree of **Master of Science** in **Biomedical Engineering, Middle East Technical University** by,

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# ABSTRACT

## SYNTHESIS OF PEPTIDE-FULLERENOL CONJUGATE AND PEPTIDE HYDROGELS FOR DRUG DELIVERY

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September 2022, 59 pages

In past decades, peptides were examined thoroughly and their use for biomedical applications enhanced largely. Peptides can be used in different forms such as self-assembly hydrogels, scaffolds, nanotubes, etc. These forms benefit various applications such as developing drugs, targeted drug delivery, wound dressings, and tissue engineering. Peptide hydrogels are used mostly for these applications that were mentioned previously. In addition, peptides can have antimicrobial properties depending on their sequence. Antimicrobial peptides (AMP) have a wide range of use in health care, animal husbandry, agriculture, aquaculture, etc.

In this thesis, Ac-$(FKFE)_2$, Ac-$(FKFE)_3$, and Ac-IKYLSVN-$NH_2$ peptides were synthesized based on the literature by using the Solid Phase Peptide Synthesis method. Then, gel formation of the peptides was examined in different solvents and solutions such as water, phosphate buffer saline (PBS), NaCl solution, and cell medium. Successful gel formation was observed for the peptide Ac-$(FKFE)_3$, and Ac-IKYLSVN-$NH_2$. It has been stated in the literature that Ac-IKYLSVN-$NH_2$ has an antimicrobial effect. Thus, biological studies were only conducted for Ac-IKYLSVN-$NH_2$ to examine antimicrobial susceptibility and drug release capability of the peptide.

Fullerenols are water-soluble fullerene derivatives which can be functionalized by amino acids and peptides to achieve desired properties. A fullerenol-peptide conjugate was tried to be synthesized and characterized using the peptide Ac-IKYLSVN-$NH_2$.

Keywords: SPPS, peptide hydrogels, antimicrobial peptides, AMP, fullerenol-peptide conjugate

# ÖZ

## FULLERENOL-PEPTİT KONJUGATININ VE PEPTİT HİDROJELLERİN İLAÇ TAŞIYICI SİSTEMLER İÇİN SENTEZİ

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Yüksek Lisans, Biyomedikal Mühendisliği
Tez Yöneticisi: Doç.Dr. Salih Özçubukçu

Eylül 2022, 59 sayfa

Geçtiğimiz yıllarda peptitler üzerine kapsamlı çalışmalar yürütülmüş ve bu çalışmalarla birlikte biyomedikal alanındaki uygulamaları da büyük ölçüde geliştirilmiştir. Peptitler, kendiliğinden oluşan hidrojeller, yapı iskeleleri, nanotüpler, vb. gibi farklı formlarda kullanılabilir. Bu formlar, ilaç geliştirme, hedefe yönelik ilaç dağıtımı, yara sargıları ve doku mühendisliği gibi çeşitli uygulamalarda fayda sağlar. Bahsedilen bu uygulamalar için çoğunlukla peptit hidrojeller kullanılmaktadır. Ek olarak peptitler, dizilimlerine bağlı olarak antimikrobiyal özelliklere sahip olabilir. Antimikrobiyal peptitler (AMP) sağlık, hayvancılık, tarım, su ürünleri yetiştiriciliği vb. alanlarda geniş bir kullanım alanına sahiptir.

Bu tezde öncelikle Ac-$(FKFE)_2$, Ac-$(FKFE)_3$, ve Ac-IKYLSVN-$NH_2$ peptitleri Katı Faz Peptit Sentezi yöntemine göre sentezlenmiştir. Daha sonra su, fosfat tamponlu tuzlu su (PBS), NaCl solüsyonu ve hücre besi yeri gibi farklı çözücü ve solüsyonlarda peptitlerin jel oluşumu incelenmiştir. Ac-$(FKFE)_3$ ve Ac-IKYLSVN-$NH_2$ peptitlerinin başarılı bir şekilde jel oluşturduğu gözlemlenmiştir. Ac-IKYLSVN-$NH_2$'nin antimikrobiyal etkiye sahip olduğu literatürde belirtilmektedir. Ac-IKYLSVN-$NH_2$ için peptitin antimikrobiyal duyarlılığını ve ilaç salma kapasitesini incelemek için biyolojik çalışmalar yürütülmüştür.

Fullerenoller, suda çözünebilen fulleren türevleri olarak istenen özellikleri elde etmek için amino asitler ve peptitler tarafından türevlendirilebilirler. Peptit sentezine ek olarak, Ac-IKYLSVN-$NH_2$ peptiti ile bir fullerenol-peptit konjugatı sentezlenmeye ve karakterize edilmeye çalışılmıştır.

Anahtar Kelimeler: SPPS, peptit hidrojel, antimikrobiyal peptit, AMP, fullerenol-peptit konjugatları

*To everyone who believed in me*

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| | |
|---|---|
| ACN | : Acetonitrile |
| AMP | : Antimicrobial Peptides |
| CCC | : Critical Coagulation Concentration |
| DAD | : Diode Array Detector |
| DCM | : Dichloromethane |
| DIEA | : *N,N*-Diisopropylethylamine |
| DMAP | : *N,N*-Dimethyl Aminopyridine |
| DMEM | : Dulbecco's Modified Eagle Medium |
| DMF | : Dimethylformamide |
| DMSO | : Dimethylsulfoxide |
| Fmoc | : Fluorenylmethyloxycarbonyl |
| HBTU | : *N,N,N′,N′*-Tetramethyl-O-(1H-benzotriazol-1-yl)uranium hexafluorophosphate |
| HFP | : 1,1,1,3,3,3-Hexafluoro-2-propanol |
| HPLC | : High Performance Liquid Chromatography |
| LBB | : Luria Bertani Broth |
| LC-MS | : Liquid Chromatography–Mass Spectrometry |
| NMR | : Nuclear Magnetic Resonance |
| PTFE | : Polytetrafluoroethylene |
| RT | : Retention Time |

SEM : Scanning Electron Microscope

TBAH : Tetrabutylammonium hydroxide

*t*-Boc : *tert*-butyloxycarbonyl

TFA : Trifluoroacetic acid

TIPS : Triisopropylsilane

TSB : Tryptic Soy Broth

# CHAPTER 1

# INTRODUCTION

## 1.1 Peptides

The building blocks of the peptides are called amino acids. Although there are hundreds of amino acids found in nature, only about 20 of them (Table 1) are considered as the main blocks of all the proteins found in the human body and most other forms of life.[1] Asparagine was the first amino acid discovered in 1806 while threonine was the last of the 20 in 1938.[2]

Table 1: List of 20 amino acids.[2]

| Amino Acid | Single Letter Code | Three Letter Code |
|---|---|---|
| Alanine | A | Ala |
| Arginine | R | Arg |
| Asparagine | N | Asn |
| Aspartic acid | D | Asp |
| Cystine | C | Cys |
| Glutamic acid | E | Glu |
| Glutamine | Q | Gln |
| Glycine | G | Gly |
| Histidine | H | His |
| Isoleucine | I | Ile |
| Leucine | L | Leu |
| Lysine | K | Lys |
| Methionine | M | Met |
| Phenylalanine | F | Phe |
| Proline | P | Pro |
| Serine | S | Ser |
| Threonine | T | Thr |
| Tryptophan | W | Trp |
| Tyrosine | Y | Tyr |
| Valine | V | Val |

Amino acids have amino (-$NH_2$), carboxy (-COOH), and side group (-R) which are bonded to the same carbon atom as illustrated in Figure 1. However, what makes them different from each other is the side group. Side groups, which are denoted as R, have various sizes, structures, and electric charges.[2] They determine the polarity, hydrophobicity, or electrical charge of the amino acids.

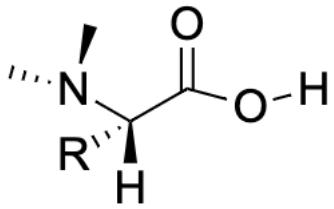


Figure 1: Basic structure of an amino acid.

Peptides are defined as polymers of amino acids bonded by peptide bonds. The carboxy group of one amino acid and the amino group of the following amino acid are bonded via the dehydration reaction.[1] Traditionally, peptides are defined as molecules with less than 50 amino acids, after 50 amino acids they are named proteins.

Peptides are used as drugs since the discovery of insulin in 1921.[3] Following this discovery, more than 80 peptide-based drugs were approved worldwide.[4] In the 21$^{st}$ century, developments in structural biology, recombinant biologics, synthetic and analytical technologies speeded the progress of peptide-based drugs, which cure diseases such as microbial, metabolic, cardiovascular, and oncological.[4]

Besides developing drugs, peptides are used for producing biomaterials such as hydrogels, scaffolds, antimicrobial coatings, and wound dressings. The comparison of peptides to synthetic or natural sources is demonstrated in Table 2. Peptide-based biomaterials can contain desired properties of both synthetic and natural biomaterials.[5] For instance, a biomaterial with low toxicity and high bioactivity can be achieved by tuning the peptide.

Table 2: Comparative advantages and challenges of differing classes of biomaterial scaffolds.[5]

| | Synthetic Biomaterials | Natural Biomaterials | Protein-engineered Biomaterials |
|---|---|---|---|
| **Toxicity** | Can be high | Low | Low |
| **Tunability** | High | Low | High |
| **Yield** | High | Low | Low |
| **Bioactivity** | Can be low | High | High |

Various types of peptide-based biomaterials are designed to resist different stimuli such as temperature, oxidative conditions, pH, or another biomolecule.[6] Several structures that can be achieved by self-assembly of peptides are shown in Figure 2.

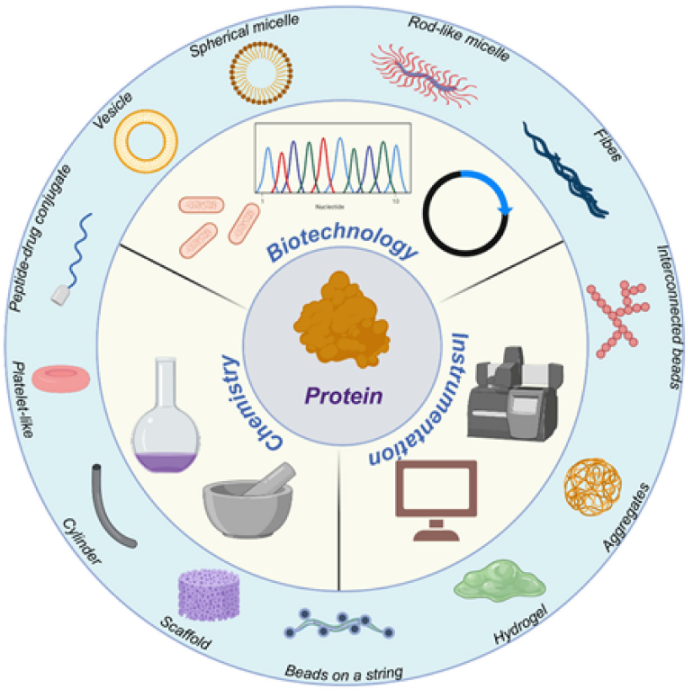


Figure 2: Recent advances in the engineering of the peptide-based biomaterials[6]

In this thesis, antimicrobial peptides, peptide hydrogels, and fullerenol-peptide conjugates were studied. Thus, they were explained in detail below.

## 1.2 Antimicrobial Peptides

Antimicrobial peptides (AMPs) are proteins with a low molecular weight that have broad antimicrobial activity against bacteria, viruses, and fungi.[7] These positively charged, evolutionarily conserved peptides typically have both a hydrophobic and a hydrophilic side, allowing them to be soluble in an aqueous medium and penetrating through lipid-rich membranes.[7] Once inside the target microbial membrane, the peptide kills target cells via various mechanisms.

The Antimicrobial Peptide Database (APD) contains 3425 antimicrobial peptides from six life kingdoms (385 isolated/predicted bacteriocins/peptide antibiotics from bacteria, 5 from archaea, 8 from protists, 25 from fungi, 368 from plants, and 2489 from animals, including some synthetic peptides) with numerous activities such as antibacterial, anti-endotoxin, anti-HIV, anti-MRSA, etc.[8]

Antimicrobial peptides can be classified on the basis of their source, activity, or structure. Subclassifications of AMPs are demonstrated in Figure 3.

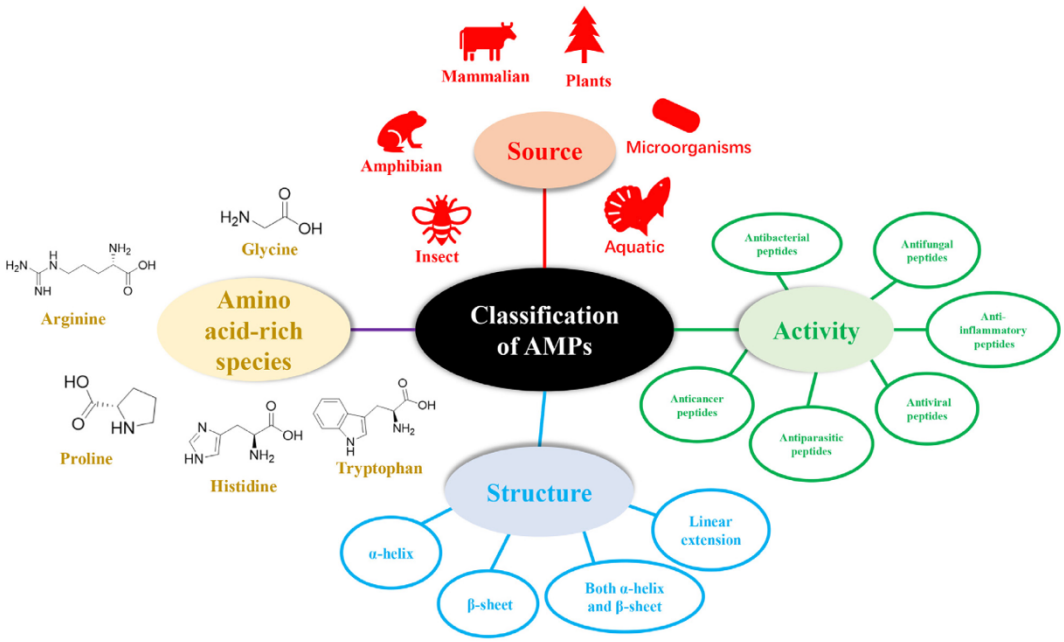


Figure 3: Classification of antimicrobial peptides.[9]

### 1.2.1 Applications of Antimicrobial Peptides

AMPs are used in several areas like health care, animal husbandry, agriculture, aquaculture, etc. However, in this part, biomedical and pharmaceutical applications of AMPs were focused on. Within the immune system, AMPs perform a variety of biological functions such as cell recruitment, speeding wound healing, killing cancer cells, inducing or modulating pro-inflammatory responses, accelerating cell proliferation, and altering gene expression.[10] Hence, they can be used for a wide range of diseases.

AMPs can overcome antimicrobial resistance, which is a defense mechanism developed by microbes against antimicrobials.[11] Antimicrobial resistance occurs when conventional antimicrobial drugs like antibiotics are misused. AMPs can overcome this; thus, they and their conjugates are used to treat microbes that are resistant to conventional drugs.

Table 3: Representative examples of antimicrobial peptides. [12]

| AMP | Discovery | MW (Da) | Bacterial strain[a] | Specificity[b] | Structure[c] |
|---|---|---|---|---|---|
| Nisin A | 1928 | 3354.1 | G(–) | *E. coli*, *S. aureus* | Polycyclic CP |
| Gramicidin S | 1944 | 1140.7 | G(+), G(–) | *S. aureus*, *E. coli* | Polycationic CP |
| Polymyxin | 1947 | 1203.5 | G(–) | *K. pneumonia*, *A. baumannii* | Polycationic CP |
| Daptomycin | 1986 | 1619.7 | G(+) | MRSA, VRE | CP lipopeptide |
| Teixobactin | 2015 | 1242.5 | G(+) | MRSA, VISA | CP |
| Melittin | 1967 | 2846.5 | G(+) | MRSA | α-helical CAP |
| Magainin | 1987 | 2409.9 | G(+), G(–) | *E. coli* | α-helical CAP |
| Cathelicidin (LL-37) | 1996 | 4493.3 | G(–) | *E. cloacae*, *K. pneumonia*, *P. aeruginosa*, | α-helical CAP |
| Buforin | 1996 | 2434.9 | G(+), G(–) | *E. coli* | α-helical CAP |

[a]G(+), gram positive, G(–), gram negative.
[b]MRSA, methicillin-resistant *Staphylococcus aureus*; VISA, vancomycin-intermediate-susceptible *Staphylococcus aureus*, *K. pneumonia* (*Klebsiella pneumonia*), *A. baumannii* (*Acinetobacter baumannii*), *E coli* (*Escherichia coli*), *S. aureus* (*Staphylococcus aureus*), *P. aeruginosa* (*Pseudomonas aeruginosa*); VRE, vancomycin-resistant *enterococci*.
[c]CAP, cationic amphipathic peptide; CP, cyclic peptide.

Numerous antimicrobial peptides have been discovered since 1928 and so much more are in clinical trials.[12] Some of the AMPs and their specificities are shown in Table 3. The positive charge of AMPs interacts with acidic polymers on the outer membrane of the bacteria which helps them to accumulate on the cell membrane. Then, AMPs can enter the bacteria cell by self-promoted uptake.[13] They whether kill the bacteria directly by cell membrane disruption or by attacking internal targets.[13]

For instance, Cao et al. prepared an injectable AMP with Ac-RKKWFW-$NH_2$ and examine its antimicrobial ability against several pathogens.[14] After zone of inhibition study, they concluded that the AMP can kill *C. albicans*, *S. aureus* and *E. coli*.[14] Other than using AMPs as drugs, implants can be coated with them to prevent biofilm formation. There are several routes to apply coatings such as contact-killing surfaces, polymer brush coatings, surface tethering, and binding domain.[15] For instance, coating of antimicrobial peptide P15-CSP both prevents biofilm activity and accelerates mesenchymal human cell attachment and spread.[16]

In literature, it has been stated that the Ac-IKYLSVN-$NH_2$ peptide has an antimicrobial effect. Zhao et al. studied the Ac-IKYLSVN-$NH_2$ peptide hydrogel and load it with glucose oxidase, which reduces glucose in the blood, to observe dual activity in diabetic wounds.[17]

## 1.3 Peptide Hydrogels

Hydrogels are three-dimensional, hydrophilic polymer networks that can absorb water or biological fluids up to 1000 times their dry weight in water or biological fluids.[18] Hydrogels are insoluble mainly because of cross-links between the components of the polymeric network.[18] Polymeric networks can be constructed by both natural and synthetic resources. Hyaluronic acid, chitosan, heparin, fibrin, and alginate are examples of natural polymers that can form hydrogels while poly(ethylene glycol), poly(vinyl alcohol), and poly(hydroxyethyl) are examples of synthetic ones.[19]

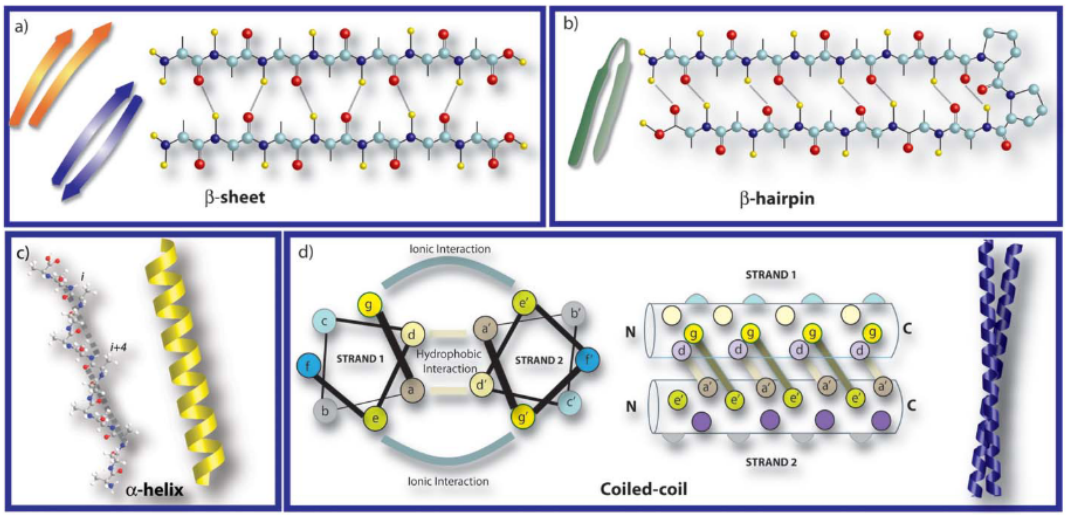


Figure 4: Schematic illustration of the molecular self-assembly involved in the formation of different secondary structures peptides: (a) ß-sheet, (b) ß-hairpin, (c) α-helix, and (d) coiled-coil.[20]

If hydrogen bonding, π-π stacking, or electrostatic interactions are present, small molecules can self-assemble to form hydrogels.[21] Natural amino acids contain all of the above-mentioned essential properties that aid self-aggregation.[20] Further, proper amino acid sequencing of the peptide could easily establish self-assembly. The various self-assembly ways that peptides can form are shown in Figure 4.

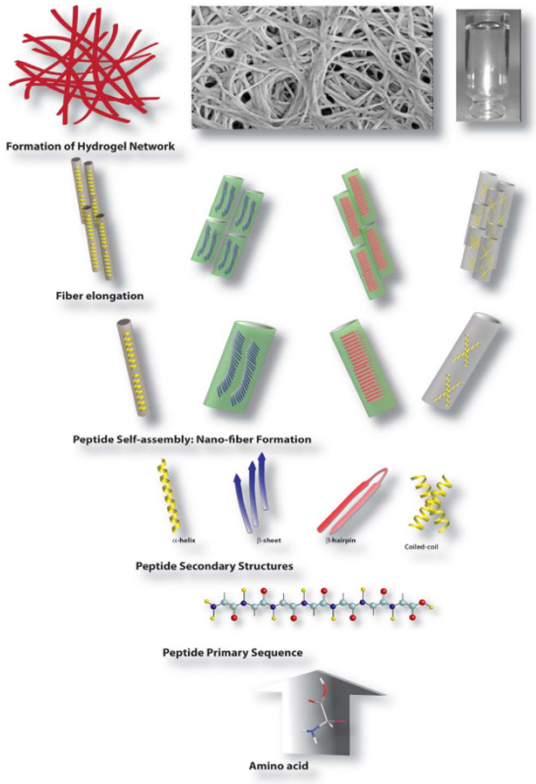


Figure 5: Simplified schematic illustrations of the hierarchical self-assembly process involved in the formation of hydrogel from peptide molecules.[20]

How peptides behave based on their secondary structures and form hydrogels are demonstrated in Figure 5. Forming peptide-based hydrogels that are significantly better designed and regulated are possible by understanding both secondary structures and the hierarchical self-assembly pathway.

### 1.3.1 Applications of Peptide-based Hydrogels

Peptide hydrogels are used widely in a lot of areas such as the food industry, tissue, and biomedical engineering, pharmacy, cosmetics, and environmental engineering.[20] Peptide-based hydrogels have advantages over other natural and synthetic-based materials as described in Table 2. For instance, synthetic self-assembling peptide biomaterials may help overcome batch inconsistency issues of natural materials

having more accurate reproducibility rates than mimics of tissues.[22] Thus, more efficient treatment can be offered to the patients.

Even if the applications of peptide-based hydrogels are narrowed down to drug delivery, numerous applications can be exhibited. Some hydrogels are sensitive to environmental stimuli and the human body has some of these like temperature and pH level. Thus, peptide-based hydrogels that respond to these stimuli are suitable for drug delivery applications.[23] In addition, they can be responsive to light, pressure, electrical current, or specific compounds such as glucose or antigens, which make them an appropriate fit for biosensors.[23,24] They are also superior to conventional drug delivery systems with their controlled degradability, adjustable sustained release, and tunability.[25] Hence, injectable hydrogels and hydrogel vesicles are used for drug delivery.

## 1.4 Solid Phase Peptide Synthesis

The first synthetic peptide (glycylglycine) was synthesized by Emil Fischer and Ernest Fourneau in 1901.[26] However, the conventional methods for synthesizing peptides were very time-consuming and complicated. Thus, progress in peptide chemistry was extremely slow until Solid Phase Peptide Synthesis (SPPS) was discovered in 1963 by R.B. Merrifield.[27] The peptide chain is synthesized gradually on the polymer resin in the SPPS method. The advantages of this method are effortless purification, fast production, automation, and being exemplary in large peptide synthesis.[28] Thus, nowadays SPPS is the number one method employed for peptide synthesis both in academia and industry.[29]

Synthesizing peptides on the resin has one very significant advantage: the resin's insolubility, which provides less complex purification steps. In other words, washing with DMF and filtration can be used to remove unreacted compounds that can cause impurities. The most advantageous part of the method is the immobilization of the peptide during the synthesis which provides elimination of unwanted compounds.[29]

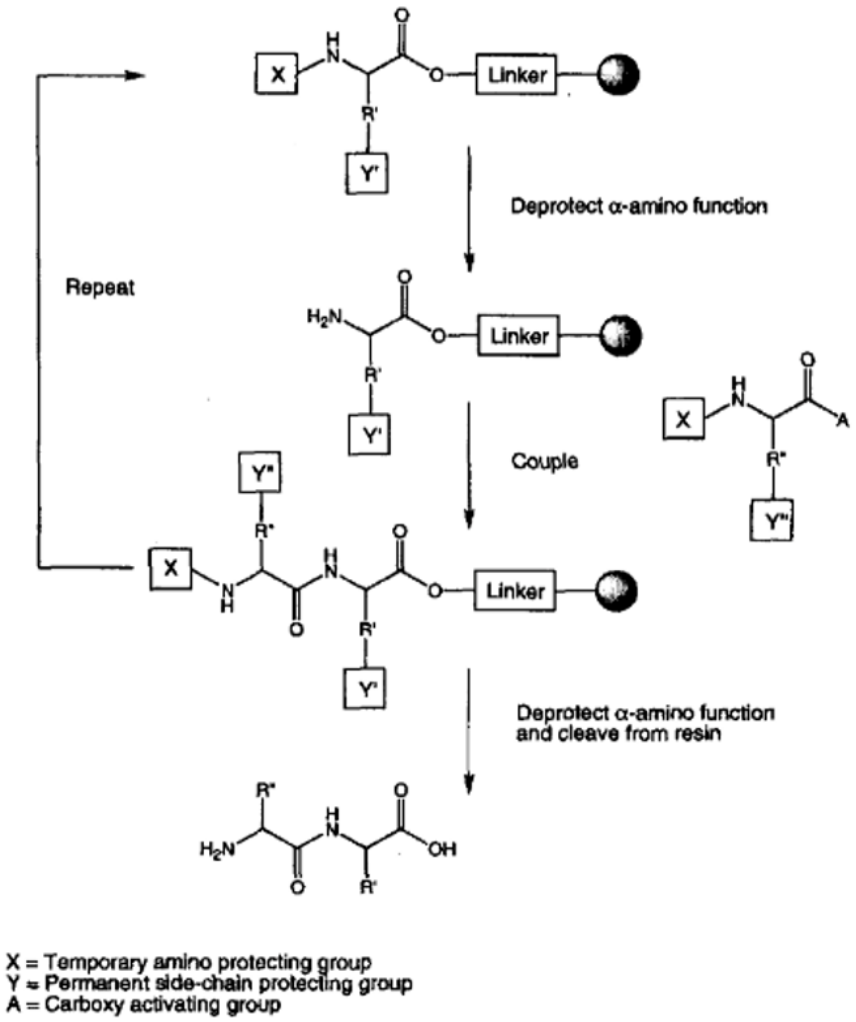


Figure 6: Representation of SPPS.[30]

As in Figure 6, the first amino acid is bonded covalently to polymer resin which is the solid backbone. In SPPS, synthesis takes place from C-terminus to N-terminus on contrary to the ribosome.[29] While the C-terminus of the amino acid is bonded to the resin, the other side of it (N-terminus with Fmoc protection) is used to bond another amino acid to this one. Thus, the peptide chain can be synthesized by repeating these steps. Between each deprotection and coupling step, the resin is rinsed with DMF thoroughly. When the desired peptide sequence is obtained, a cleavage solution is used to cleave the peptide from the resin.

There are two protection groups used in the SPPS method, Fmoc, and *t*-Boc. In the Fmoc and *t*-Boc methods piperidine and TFA are used for deprotection of N-

terminus, respectively.[29] The advantage of Fmoc over *t*-Boc is its cleavage solution. In the Fmoc method, TFA is used as a cleavage solution while in *t*-Boc highly toxic hydrogen fluoride is used.[31] Hence, Fmoc protection is used significantly more.

### 1.4.1 Solid Phase Peptide Synthesis with Microwave Synthesizer

The first trials of microwave in peptide synthesis reported in 1992 belong to Yu.[32] He synthesized two peptide sequences, GFGVAVLG and GVGFVIG.[32] Synthesis route does not change compared to the manual synthesis however, there are lots of advantages using microwave synthesizer such as higher purity and yield, shorter reaction times, less usage of solvent and reagents, and improved reproducibility. On the other hand, cysteine and histidine show racemization due to high temperatures. Thus, coupling of the peptide sequences containing them should be performed maximum of 50 ºC.[33] In this thesis, the second batch of Ac-$(FKFE)_3$ and four batches of Ac-IKYLSVN-$NH_2$ are synthesized in a microwave synthesizer at 75 ºC.

## 1.5 Fullerene

Allotropy is defined as the co-existence of more than one form of an element in the same physical state.[34] Carbon is one of these elements. It exists in nature as diamond, graphite, and fullerene forms.[35] Fullerene was discovered in 1985 by Kroto et al. while they are trying to understand the underlying mechanism of long-chain carbon molecules. Buckminsterfullerene which has a form of football is the most known one in the fullerene family thanks to its abundance in nature.[36] Fullerenes have a wide range of biological applications. They can use as neuroprotective agents, antioxidants, and enzyme mimics while it is detected that they also have antimicrobial activity.[37] For instance, Prato et al. tested one of fullerene derivatives' antimicrobial ability on *Mycobacterium* spp. by spreading the fullerene derivate on Petri dishes filled with bacteria and observed that the derivate inhibits proliferation of the bacterium.[38]

### 1.5.1 Fullerenol

Fullerene has lots of derivates and fullerenol is the most known one. It can be obtained by hydroxylation of the fullerene in various ways.[39] Derivatizing fullerene with large functional groups is complicated thus fullerenol can be used instead of it. The synthesis route and different derivatives of fullerenol are presented.

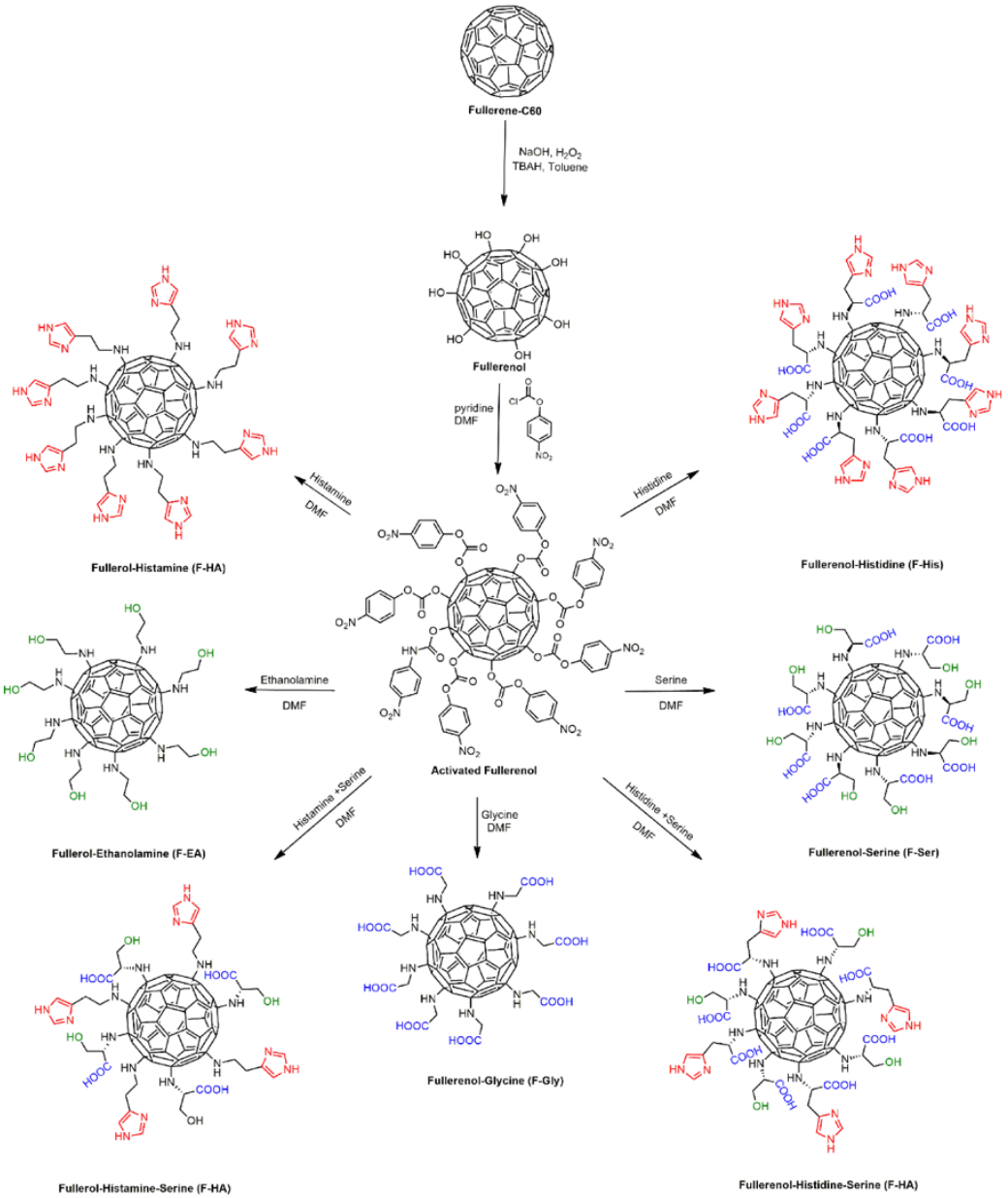


Figure 7: Synthesis route and illustration of all fullerene derivatives.[40]

Fullerenol has an antiviral and cytotoxic activity which makes them useful as a drug alternative as well as they can be used as a drug and gene delivery agent.[41] For instance Jiao et al. performed animal studies on mice to evaluate the anti-cancer ability of fullerenol on breast cancer and they observed that tumor shrinks directly proportional to increased dosage of fullerenol.[42]

## 1.6 Aim of the Study

Based on the literature, it is known that Ac-$(FKFE)_2$, Ac-$(FKFE)_3$, and Ac-IKYLSVN-$NH_2$ peptides can form a hydrogel.[17,43] However, their antimicrobial or drug release abilities have not been studied deeply. The aim is that to see whether Ac-$(FKFE)_2$ and Ac-$(FKFE)_3$ contributes proliferation of mammalian cells when their gel form spread on a surface. Also, it is aimed that examination of Ac-IKYLSVN-$NH_2$ peptide's antimicrobial abilities on gram positive and negative bacteria.

Thus, the peptides were synthesized according to the Solid Phase Peptide Synthesis method. Then, the gel formation of the peptides was examined in different solvents and solutions such as water, phosphate buffer saline (PBS), NaCl solution, and cell medium.

Following gelation studies, biological studies were conducted for Ac-$(FKFE)_3$ and Ac-IKYLSVN-$NH_2$. For the letter, peptide's antimicrobial susceptibility and drug release capability are examined.

In addition to peptide synthesis and their biological studies, a fullerenol-peptide conjugate was tried to be synthesized with the peptide Ac-IKYLSVN-$NH_2$ to examine. Also, the characterization and surface composition of the conjugate were studied.

# CHAPTER 2

# RESULTS AND DISCUSSION

## 2.1 Peptide Hydrogels

Three different peptides were synthesized by using the SPPS method.[27] During the studies, generally, the first batch was synthesized manually within the 0.05 to 0.1 mmol range.

### 2.1.1 Synthesis of Ac-(FKFE)$_2$

One batch of Ac-(FKFE)$_2$ was synthesized manually according to the Solid Phase Peptide Synthesis method. Rink amide resin equivalent to 0.05 mmol (152.2 mg) was used. ~26 mg crude peptide is obtained with 76.35% purity (Figure 8).

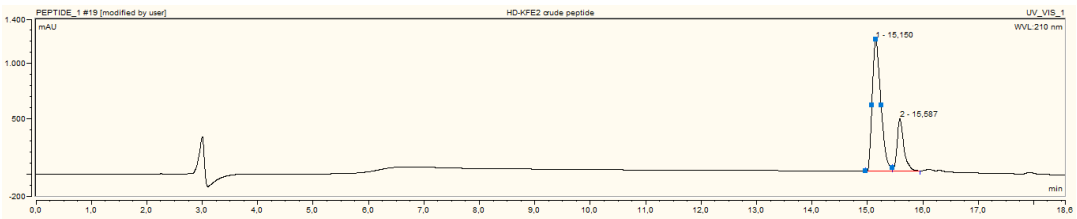


Figure 8: HPLC chromatogram of crude Ac-(FKFE)$_2$.

As represented in Figure 8, following the main peptide peak a huge impurity peak was present. Thus, purification was performed on an HPLC instrument.

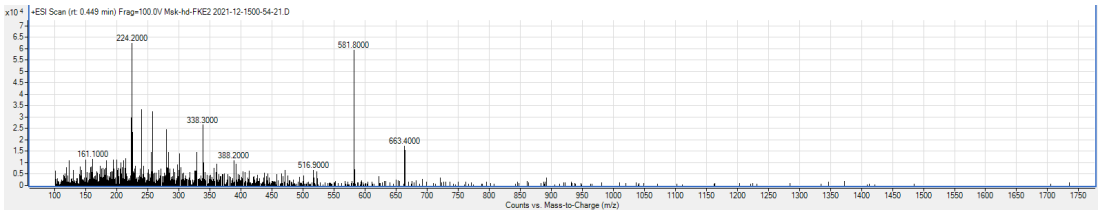


Figure 9: Mass spectrum of Ac-(FKFE)$_2$ $[M+2H]^{2+}_{calc}$ = 581.17 $[M+2H]^{2+}_{obs}$ = 581.80.

Performing a purification study with 250x10mm 5µm Thermo Scientific ODS Hypersil™ semi-preparative HPLC column, 92.2% purity was observed as in Figure 10.

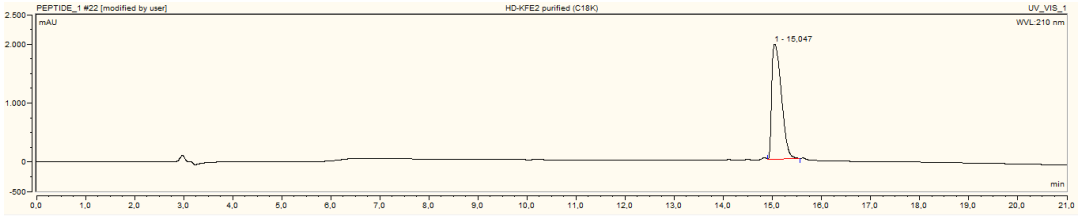


Figure 10: HPLC chromatogram of Ac-(FKFE)$_2$ at 210 nm after purification.

### 2.1.2 Gelation Study of Ac-(FKFE)$_2$

1% solutions of Ac-(FKFE)$_2$ form gel between the minimum and maximum critical coagulation concentrations which are 1 mM and 5 mM, respectively.[43] 3 mg Ac-(FKFE)$_2$ was mixed with 300 µL 2.5 mM NaCl solution and gel was formed within 5 minutes when the tube was placed in the ultrasonic bath. Then the gel form was disturbed with mechanical force by using a vortex. Following this, the tube was placed in the ultrasonic bath again for 20 minutes. Unfortunately, Ac-(FKFE)$_2$ cannot form gel once again. The tube was placed on the bench and observed one day later and the disturbed gel form was still viscous. Biological studies of the gel require it to form gel again when disturbed. Thus, further analysis was not conducted for Ac-(FKFE)$_2$.

### 2.1.3 Synthesis of Ac-(FKFE)$_3$

Two batches of Ac-(FKFE)$_3$ were synthesized. The first batch of Ac-(FKFE)$_3$ was synthesized manually according to the Solid Phase Peptide Synthesis method. Rink amide resin equivalent to 0.1 mmol (302.12 mg) was used. ~70 mg crude peptide was obtained with 77.2% purity (Figure 11).

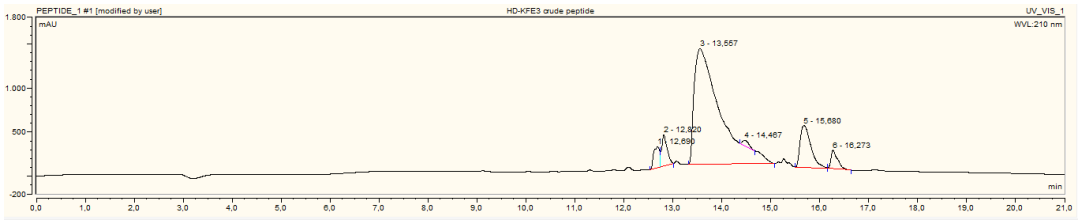


Figure 11: 1$^{st}$ batch's HPLC chromatogram of Ac-(FKFE)$_3$ at 210 nm before purification.

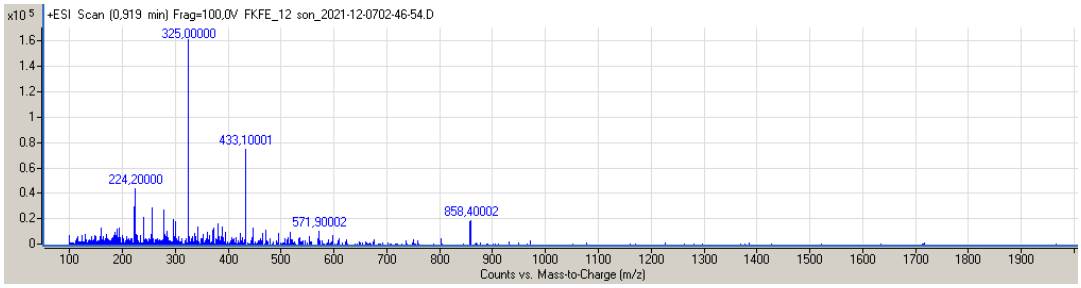


Figure 12: Mass spectrum of Ac-(FKFE)$_3$, $[M+2H]^{2+}_{calc}$ = 857.44 $[M+2H]^{2+}_{obs}$ = 858.40.

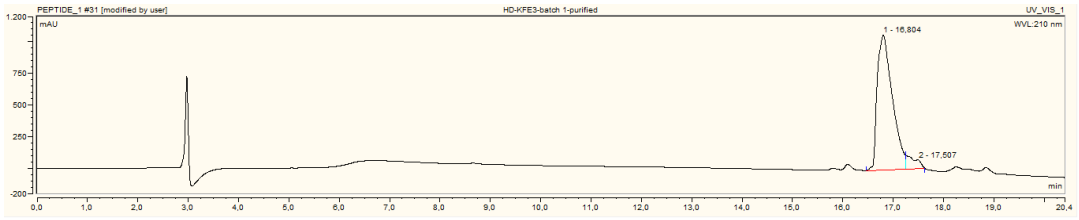


Figure 13: 1$^{st}$ batch's HPLC chromatogram of Ac-(FKFE)$_3$ at 210 nm after purification.

Performing a purification study with 250x10mm 5 µm Thermo Scientific ODS Hypersil™ semi-preparative HPLC column, 89.3% purity was observed as in Figure 13.

The second batch of Ac-$(FKFE)_3$ was synthesized in a microwave synthesizer according to the SPPS method. ~286 mg crude peptide was obtained in this batch (0.25 mmol) thus, 8 purification analysis was conducted to purify all the crude peptide. Purifications of the second batch are denoted as batch 2.1 –2.8.

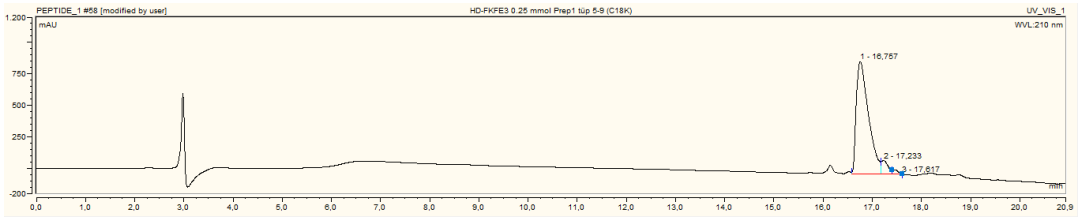


Figure 14: HPLC chromatogram of purification of batch 2.1 at 210 nm, purity 87.64%.

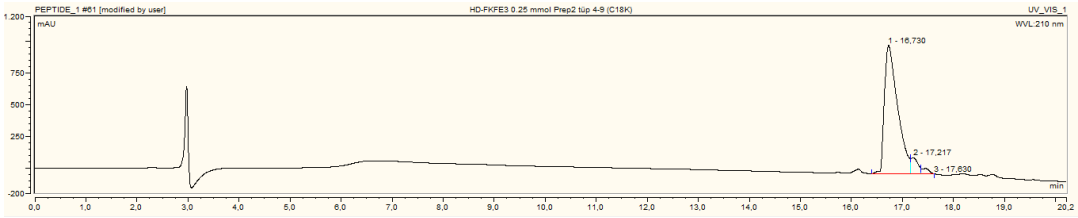


Figure 15: HPLC chromatogram of purification of batch 2.2 at 210 nm, purity 88.31%.

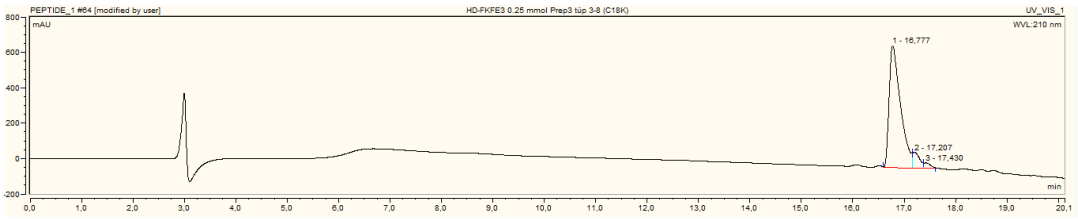


Figure 16: HPLC chromatogram of purification of batch 2.3 at 210 nm, purity 88.21%.

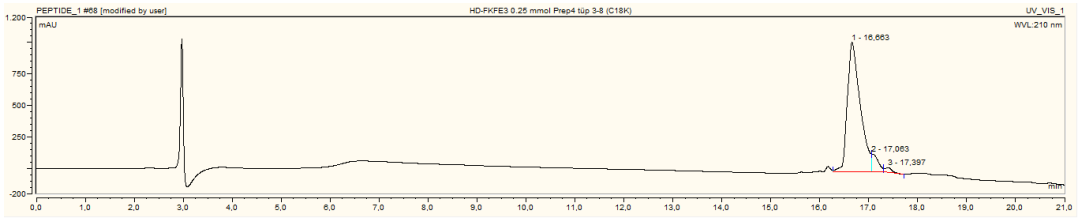


Figure 17: HPLC chromatogram of purification of batch 2.4 at 210 nm, purity 88.80%.

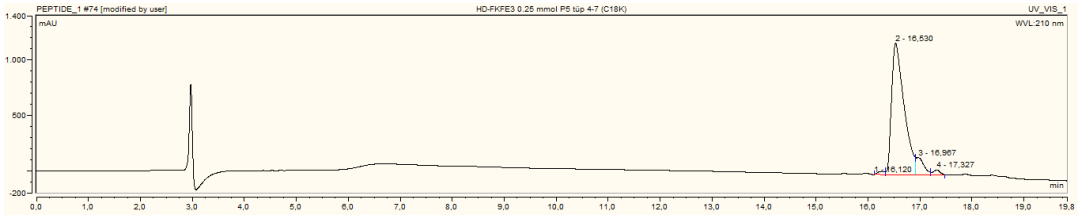


Figure 18: HPLC chromatogram of purification of batch 2.5 at 210 nm, purity 88.27%.

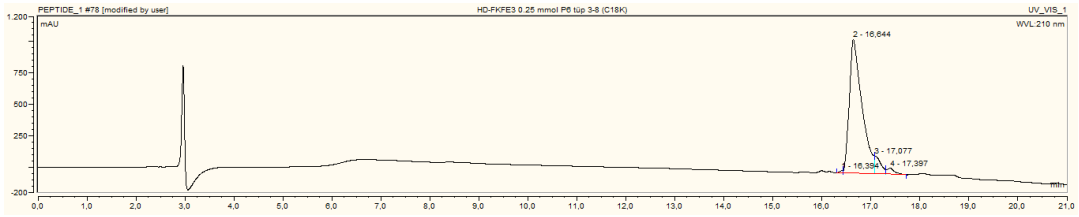


Figure 19: HPLC chromatogram of purification of batch 2.6 at 210 nm, purity 88.39%.

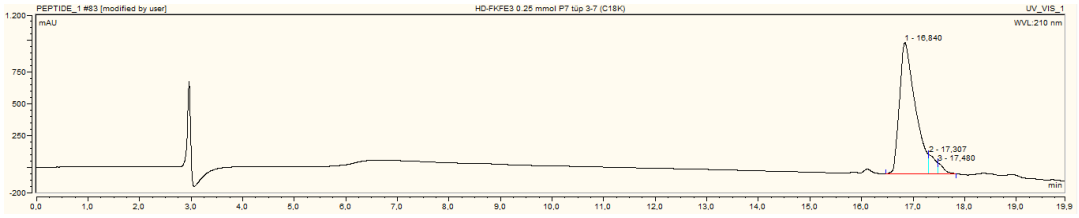


Figure 20: HPLC chromatogram of purification of batch 2.7 at 210 nm, purity 88.65%.

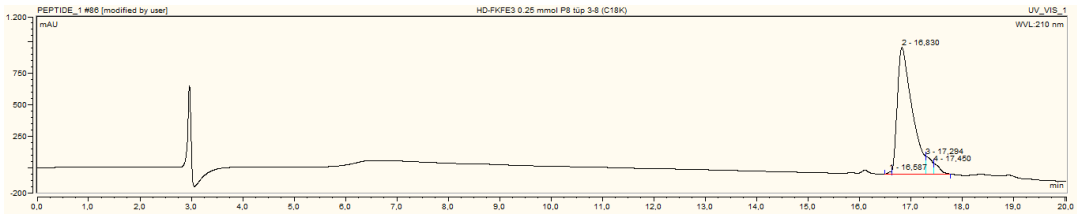


Figure 21: HPLC chromatogram of purification of batch 2.8 at 210 nm, purity 88.12%.

An average of ~88% purity was obtained in the second batch of the peptide as presented in figures between 14-21.

### 2.1.4 Gelation Study of Ac-(FKFE)$_3$

1% solutions of Ac-(FKFE)$_3$ form gel between the minimum and maximum critical coagulation concentrations which are 0.1 mM and 1 mM NaCl, respectively.[43] 3 mg Ac-(FKFE)$_3$ was mixed with 300 µL 0.5 mM NaCl solution and the gel was formed in 20 minutes when the tube was placed in the ultrasonic bath. Then the gel form was disturbed with mechanical force by using a vortex. It was observed that the gel was resistant to mechanical forces. Following the mechanical forcing of the gel, the tube was placed in the ultrasonic bath again for 20 minutes and the gel was formed once more (Figure 22).

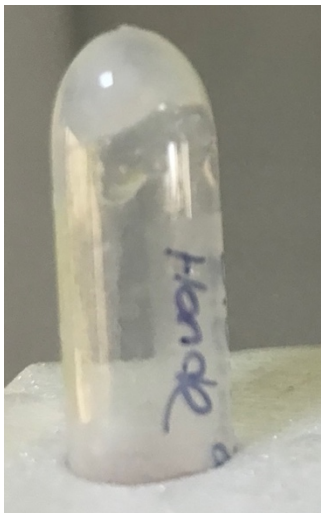

Figure 22: Gel form of Ac-(FKFE)$_3$.

### 2.1.5 Biological Study of Ac-(FKFE)$_3$

In the literature, pH for optimal growth of mammalian cells is denoted as ~7.4.[44] The pH of the 1% which was prepared with 0.5 mM NaCl solution was measured as ~2.34 which is not suitable for living cells. Thus, pH of the gel was tried to adjust ~ 7 by using 1 M NaOH. A total of 3 trials were conducted and in all trials 1% gel of the peptide prepared in 300 µL of 0.5 mM NaCl solution.

Table 4: Added NaOH amounts to Ac-(FKFE)$_3$ gel in the first trial.

| 1M NaOH added in total (µL) | pH |
|---|---|
| - | 2.34 |
| 0.5 | 2.73 |
| 1.5 | 3.24 |
| 2.5 | 3.60 |
| 4 | 6.80 |

According to the calculations, 1.57 µL of 1 M NaOH should be added to 1% Ac-(FKFE)$_3$ gel to adjust to pH ~7 for a gel prepared as 3 mg peptide in 300 µL 0.5 mM NaCl solution. In Table 4, amounts of 1 M NaOH added are shown in the first trial. In this trial, the first batch of the Ac-(FKFE)$_3$ was used.

After the addition of 1 M NaOH solution, the gel was placed in a vortex for ~1 minute and sonicated for 20 minutes in each step. In literature, it was stated that Ac-(FKFE)$_3$ can form gel between pH 3-8.[43] Parallel to this information when the pH of the gel was measured as 6.8, the gel form was still intact. On the other hand, the gel formation in its own pH was placed in 20 minutes while it took nearly two hours at pH 6.8. In the last step, the tube was sonicated for one more hour, and the tube was placed on a bench for observation of whether it would form gel again or not. After sonication, the gel was a viscous solution which indicates that the gel formation was in progress. In about two hours, the gel form was obtained once more.

In the second trial, the second batch of the Ac-(FKFE)$_3$ was used. In the light of the first pH adjustment trial, the steps were reduced to two as in Table 5. When

comparing two initial pH values, it was expected that the final pH value of the second trial would be around pH 7-7.2. However, the final pH value was measured as 8.30. Hence, the gel form was damaged.

Table 5: Added NaOH amounts to Ac-$(FKFE)_3$ gel in the second trial.

| 1M NaOH added in total (µL) | pH |
|---|---|
| - | 2.53 |
| 2 | 3.83 |
| 4 | 8.30 |

In the third trial, also the second batch of Ac-$(FKFE)_3$ was used. At the end of the three steps of pH adjustment, the final pH value of the gel was measured as 7.30 (Table 6).

Table 6: Added NaOH amounts to Ac-$(FKFE)_3$ gel in the third trial.

| 1M NaOH added in total (µL) | pH |
|---|---|
| - | 2.46 |
| 2 | 3.72 |
| 3 | 3.80 |
| 3.5 | 7.30 |

While the gel was still viscous, two wells of the tissue culture plate were filled with 100 µL of it by an automatic pipette. Then, 200 µL of DMEM which is a cell medium was added to the wells. The aim of this was to see whether the gel and DMEM are compatible for up to five days in an incubator or not.

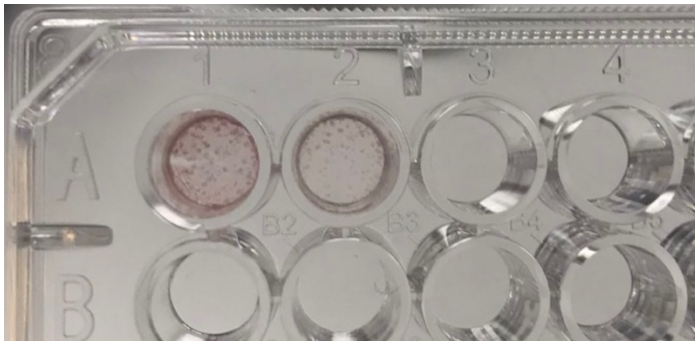


Figure 23: Dried Ac-$(FKFE)_3$ gel in TCP wells.

Unfortunately, after two days all the gel in the wells was dried even the tissue culture plate had a cap (Figure 23). Thus, no further analysis could be conducted.

## 2.2 Antimicrobial Peptides

### 2.2.1 Synthesis of Ac-IKYLSVN-$NH_2$

A total of 5 batches of Ac-IKYLSVN-$NH_2$ were synthesized. All batches except the first one were synthesized in a microwave synthesizer. Rink amide resin equivalent to 0.1 mmol (302.1 mg) was used for the first batch while 0.3 mmol (906.3 mg) was used for the rest of the batches. Due to the high purity of the Ac-IKYLSVN-$NH_2$, no further purification step was conducted.

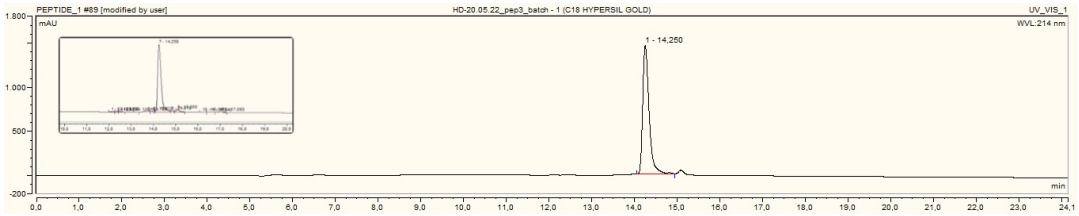


Figure 24: HPLC chromatogram of Ac-IKYLSVN-$NH_2$ at 210 nm.

The purity of the first batch of Ac-IKYLSVN-$NH_2$ was observed as 92.5% after lyophilization of the peptide (Figure 24).

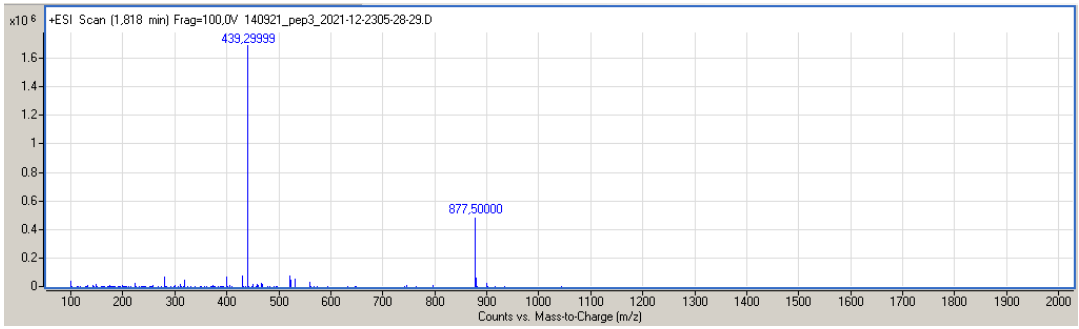


Figure 25: Mass spectrum of Ac-IKYLSVN-$NH_2$ $[M+2H]^{2+}_{calc}$ = 877.04 $[M+2H]^{2+}_{obs}$ = 877.50.

Mass analysis was only conducted for the first batch (Figure 25). The following four batches were controlled with respect to the retention time of the first batch in HPLC chromatogram.

The other four batches had lower purity than the first batch. Possible effects of lower purity could be the larger batch size and microwave synthesizing compared to the first batch. As in Figure 26, the purity of batch 2 was 83.5%.

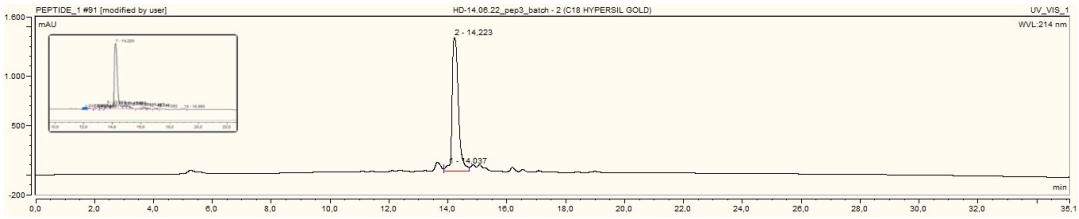


Figure 26: HPLC chromatogram of batch 2 at 210 nm.

The purity of the batch 3, 4, and 5 are 88.6%, 85.2%, and 83.3%, respectively (Figure 27,28, and 29).

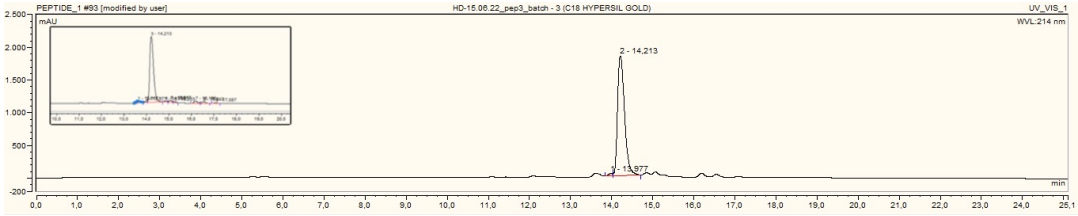


Figure 27: HPLC chromatogram of batch 3 at 210 nm.

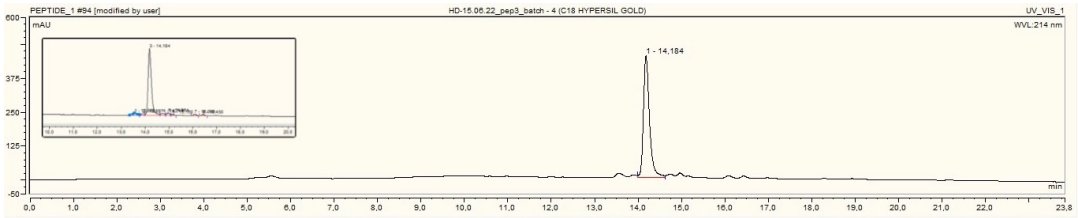


Figure 28: HPLC chromatogram of batch 4 at 210 nm.

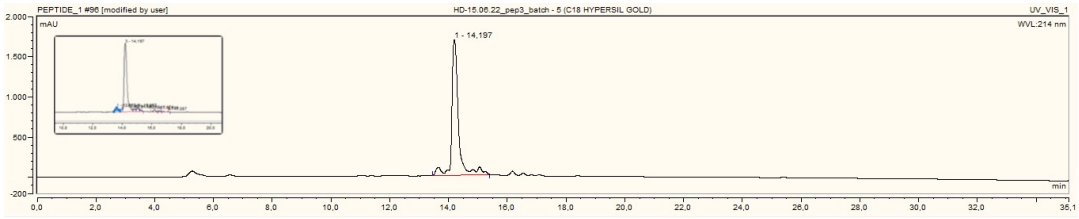


Figure 29: HPLC chromatogram of batch 5 at 210 nm.

After the assay of each batch was separately tested, all batches are mixed in a falcon tube and the peptide was lyophilized. Following lyophilization, another assay test was conducted for the peptide. The purity of the mixed batch was observed as 87.1% (Figure 30).

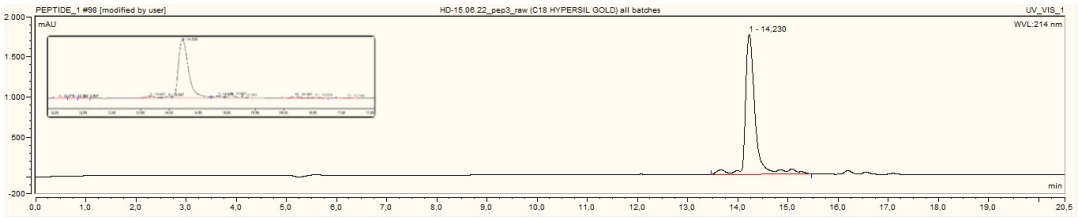


Figure 30: HPLC chromatogram of the mixed batch at 210 nm.

All assay tests were performed on Dionex Ultimate 3000 HPLC with DAD detector instrument using 150x4.6mm 5µm, C18 Thermo Scientific ODS Hypersil™ Gold HPLC column.

### 2.2.2 Gelation Study of Ac-IKYLSVN-$NH_2$

In literature, it has been stated that 1% solutions of Ac-IKYLSVN-$NH_2$ with distilled water form a gel.[17] To study different solvents for gelation, Milli-Q water, and PBS solution were used. After solvents were added, the tubes were placed in the ultrasonic bath for 15 minutes. It was observed that some of the peptides did not dissolve. The tubes are placed in the vortex for ~30 seconds following 15 minutes ultrasonic bath. 1% solutions of these solvents however could not pass the inversion test. As shown in Figure 31, 1% solution of the peptide did not form gel while 2.5% solution did with Milli-Q.

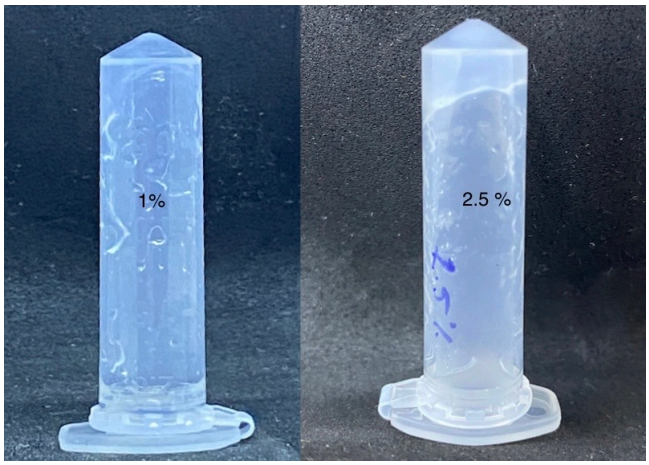


Figure 31: Inversion test of Ac-IKYLSVN-$NH_2$, Left: 1% solution with Milli-Q water, Right: 2.5% solution with Milli-Q.

In addition to these concentrations, 2%, 3%, 4%, and 5% solutions were prepared for the Kirby Bauer test with PBS. All concentrations were able to form gel successfully. Their stiffness can be observed in Figure 32 by the gels' color getting cloudier.

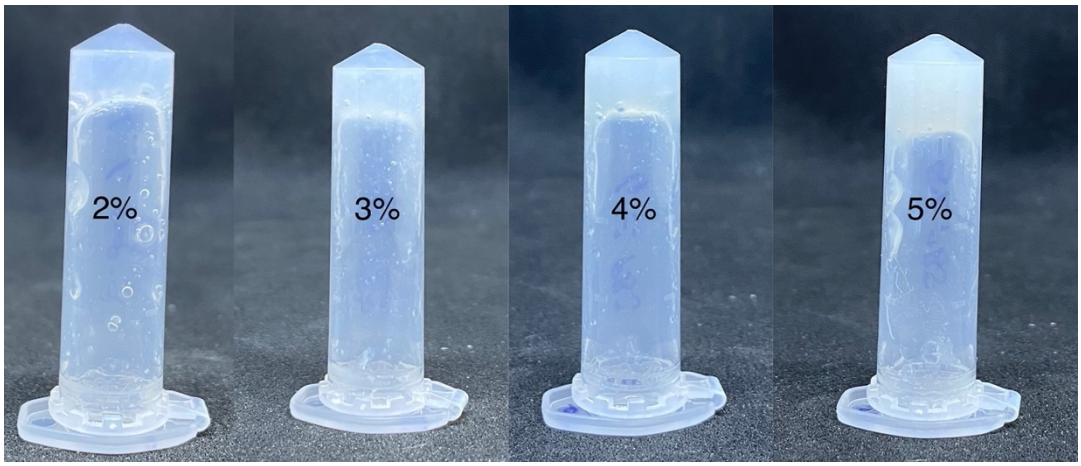


Figure 32: Ac-IKYLSVN-$NH_2$ gels in different concentrations with PBS.

Also, PBS + chloramphenicol solution was used to assess the gel's drug release capability. For this assessment, 2%, 3%, 4%, and 5% solutions were prepared with PBS + chloramphenicol solution. All concentrations formed a gel successfully (Figure 33).

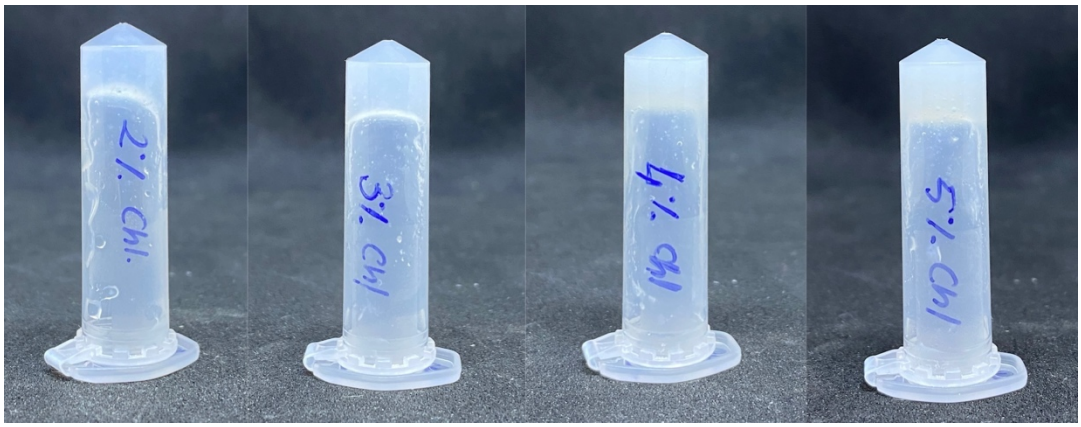


Figure 33: Ac-IKYLSVN-NH2 gels in different concentrations with PBS + chloramphenicol solution.

### 2.2.3 Biological Study of Ac-IKYLSVN-$NH_2$

#### 2.2.3.1 Compatibility Test

When Ac-$(FKFE)_3$ gel was observed as non-compatible with DMEM in an incubator, the same procedure was applied for the Ac-IKYLSVN-$NH_2$ gel. Two wells of the tissue culture plate were filled with 100 µL of it by an automatic pipette. Then, 200 µL of DMEM which is a cell medium was added to the wells.

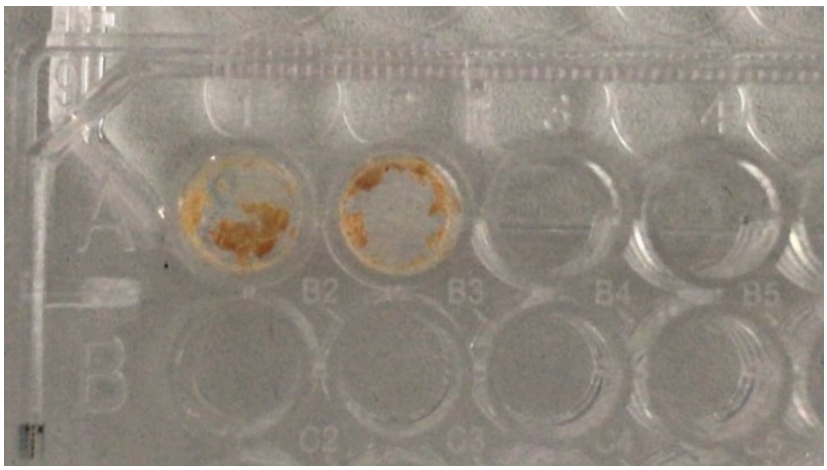


Figure 34: Dried Ac-IKYLSVN-$NH_2$ gel in TCP wells.

It was expected that the gel would be compatible with the cell medium for up to 5 days. Unfortunately, the gel dried after two days in the incubator as well as Ac-$(FKFE)_3$ gel (Figure 34). Hence, no further biological analysis can be conducted.

### 2.2.3.2 Antibacterial Study of Ac-IKYLSVN-$NH_2$

The Ac-IKYLSVN-$NH_2$ peptide was dissolved in DMEM with concentrations of 5 mg/mL and 10 mg/mL. If the Ac-IKYLSVN-$NH_2$ peptide has an antimicrobial effect, there would be zones of inhibition on the agar plate where 10 µL of the 5 mg/mL and 10 mg/mL peptide solutions were dropped, respectively. However, as can be seen in Figure 35, there were no zones on both agar plates which contain *E. coli* and *S. aureus*, respectively.

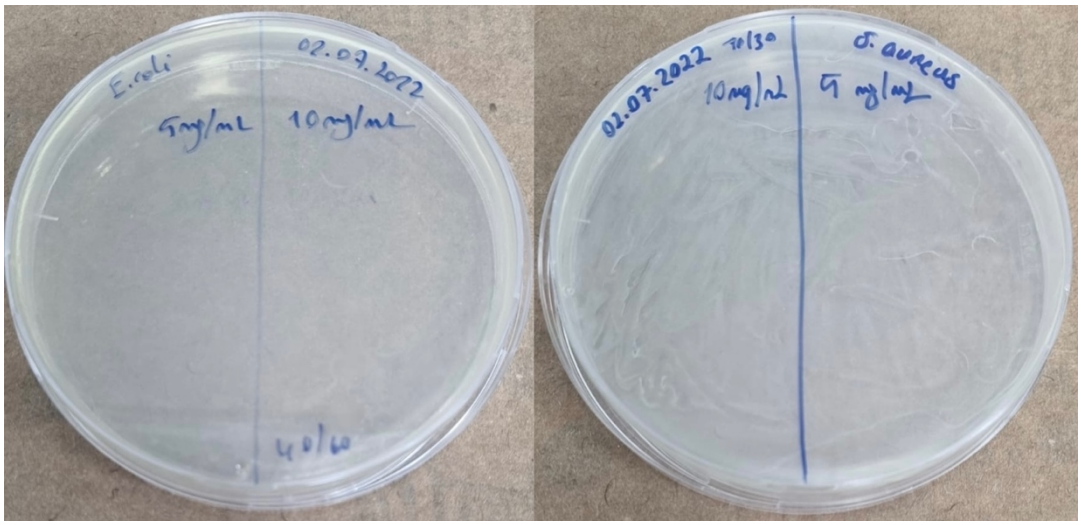


Figure 35: Agar plates treated with *E. coli* and *S. aureus.*

### 2.2.3.3 Disc Diffusion Test of Ac-IKYLSVN-$NH_2$

The gels of the peptide which were at different concentrations (2%, 3%, 4%, and 5%) with two different solutions (PBS and PBS + chloramphenicol solutions) were successfully observed and reported in title 2.2.2. After this protocol, gels were

transferred to wells on agar plates that contain *S.aureus* and *E.coli* to see whether the peptide had drug release capability.

Zone of inhibition formation was expected around the wells containing chloramphenicol which are positive control and Ac-IKYLSVN-$NH_2$ gel prepared with PBS + chloramphenicol solution. There should be no zone of inhibition around PBS (- control) and Ac-IKYLSVN-$NH_2$ gel prepared with PBS solution. Agar plates were incubated for 16 hours, and zone of inhibition sizes was measured. Then the incubation time was completed to 40 hours. All samples are displayed between figures 36 and 51.

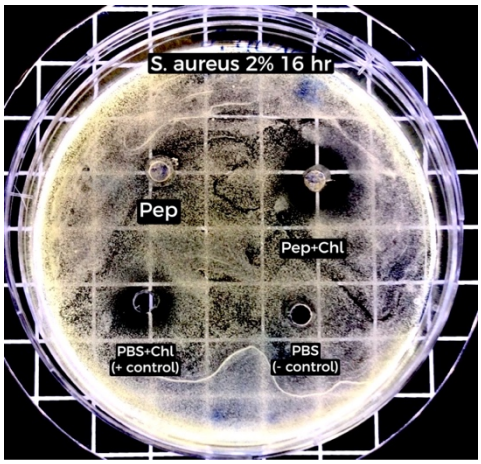


Figure 36: 2% gel at 16$^{th}$ h. on *S. aureus* agar plate.

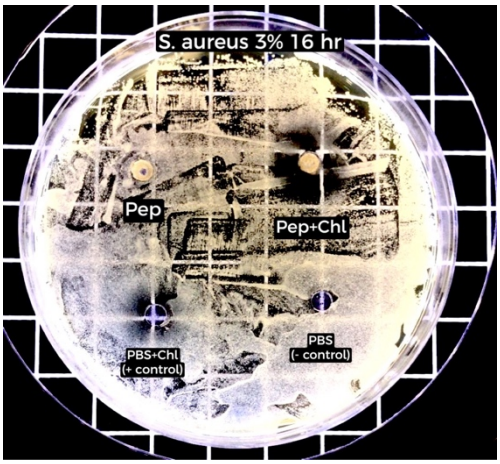


Figure 37: 3% gel at 16$^{th}$ h. on *S. aureus* agar plate.

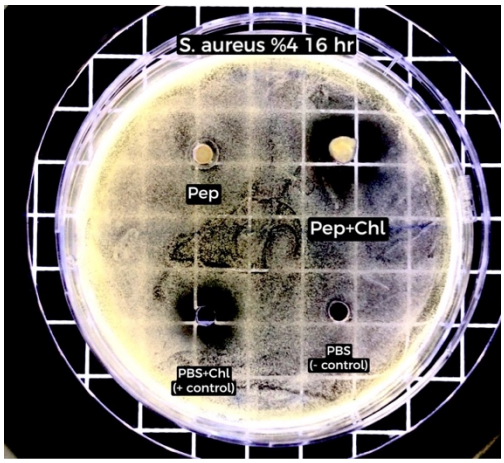


Figure 38: 4% gel at 16th h. on *S. aureus* agar plate.

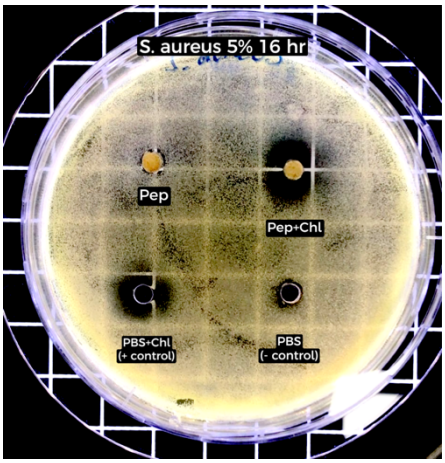


Figure 39: 5% gel at 16th h. on *S. aureus* agar plate.

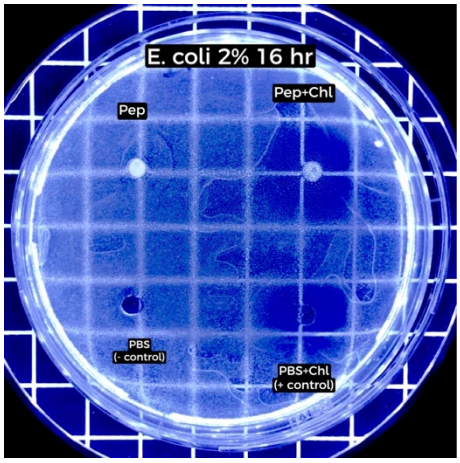


Figure 40: 2% gel at 16th h. on *E. coli* agar plate.

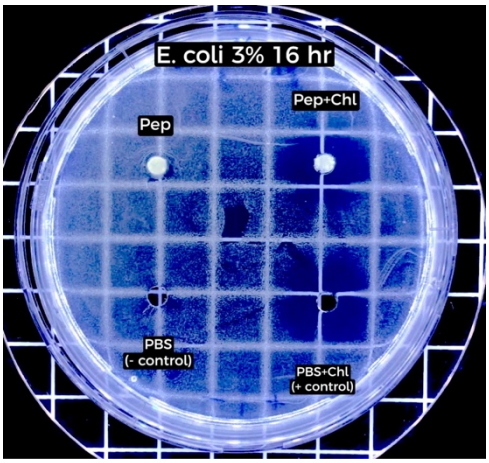


Figure 41: 3% gel at 16th h. on *E. coli* agar plate.

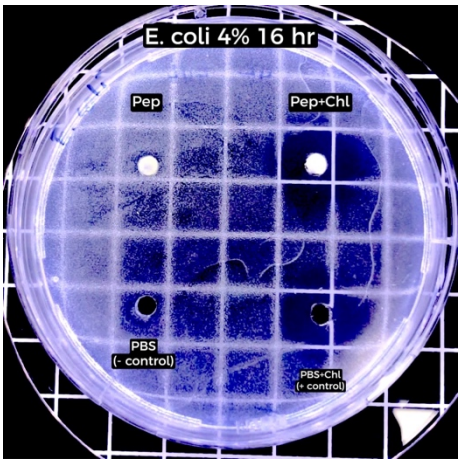


Figure 42: 4% gel at 16th h. on *E. coli* agar plate.

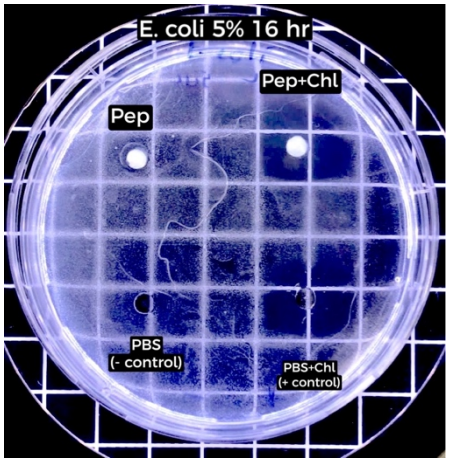


Figure 43: 5% gel at 16th h. on *E. coli* agar plate.

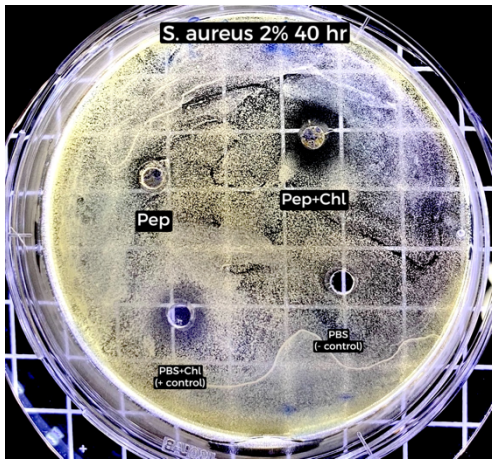


Figure 44: 2% gel at 40th h. on *S. aureus* agar plate.

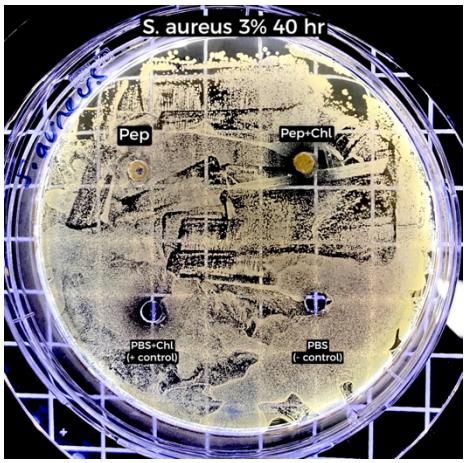


Figure 45: 3% gel at 40th h. on *S. aureus* agar plate.

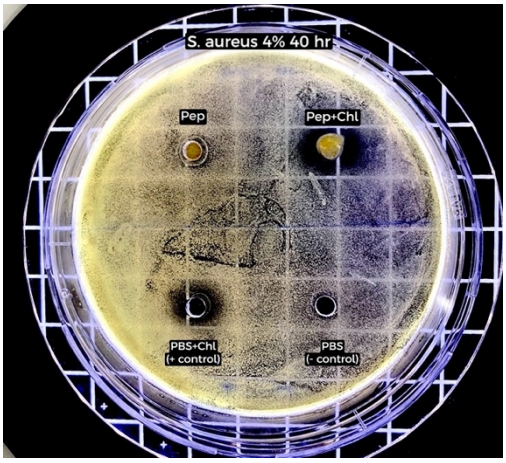


Figure 46: 4% gel at 40th h. on *S. aureus* agar plate.

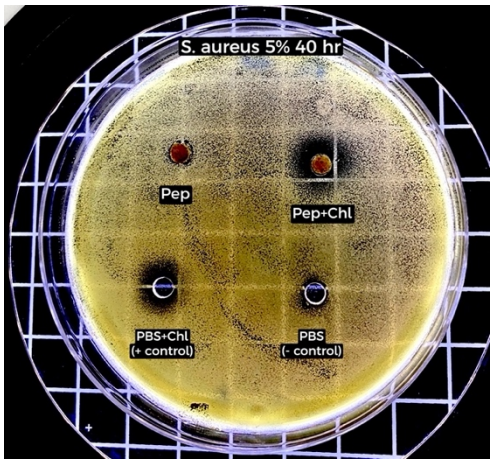


Figure 47: 5% gel at 40th h. on *S. aureus* agar plate.

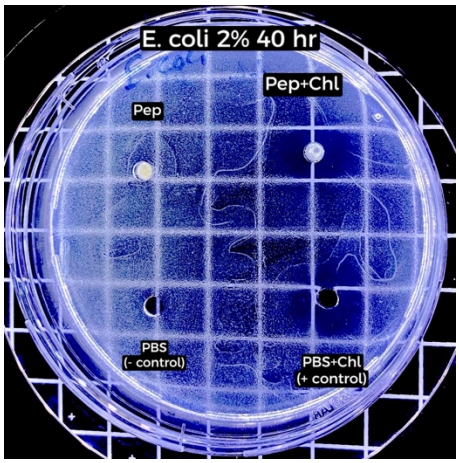


Figure 48: 2% gel at 40th h. on *E. coli* agar plate.

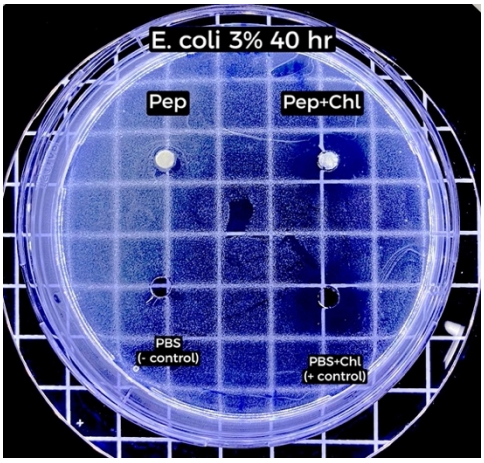


Figure 49: 3% gel at 40th h. on *E. coli* agar plate.

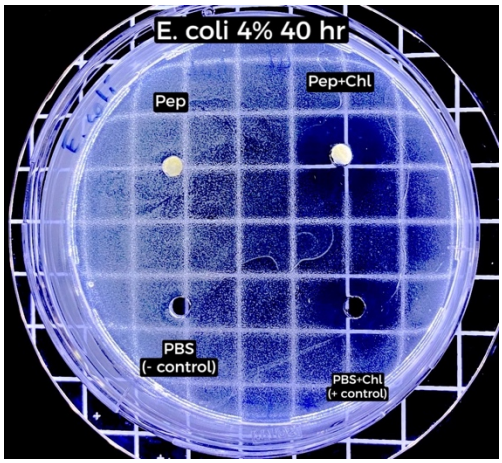


Figure 50: 4% gel at 40th h. on *E. coli* agar plate.

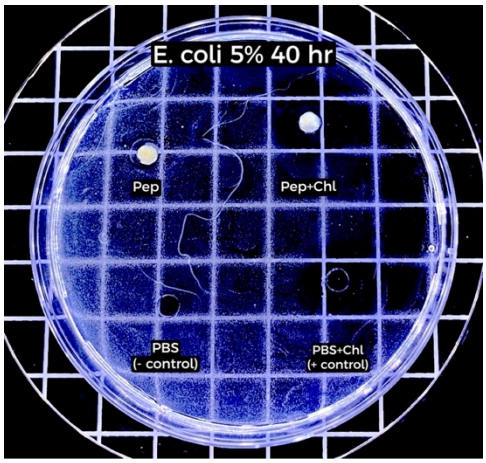


Figure 51: 5% gel at 40th h. on *E. coli* agar plate.

As shown in Figures 36-51 that all gels prepared with PBS + chloramphenicol solution forms zone of inhibition. As the negative control did not have zone of inhibition, it could be stated that the gel has drug release ability.

Table 7 and 8 displays the size differences in zone of inhibition between 16th and 40th hours of *S. aureus* and *E. coli,* respectively. The peptide+chloramphenicol solution was expected to have a larger zone of inhibition than PBS + chloramphenicol as it was a positive control. However, for *S. aureus,* the zone of inhibition of PBS + chloramphenicol was larger than the positive control (Table 7). There could be some experimental error as it is just one repetition. Thus, as future work, this study should be repeated with three replicates.

Table 7: Measured zone of inhibition diameters on *S. aureus* agar plate.

| Hour | Peptide+Chl | | Peptide | PBS + chloramphenicol | PBS |
|---|---|---|---|---|---|
| 16th hour | 2% | 1.5 | ND | 1.5 | ND |
| | 3% | 1.7 | ND | 1.1 | ND |
| | 4% | 2.1 | ND | 1.7 | ND |
| | 5% | 1.5 | ND | 1.5 | ND |
| 40th hour | 2% | 1.3 | ND | 1.0 | ND |
| | 3% | 1.4 | ND | 0.8 | ND |
| | 4% | 1.6 | ND | 1.2 | ND |
| | 5% | 1.1 | ND | 0.8 | ND |

For *E. coli,* the zone of inhibition for the positive control was larger than the peptide+chloramphenicol sample as expected (Table 8).

Table 8: Measured zone of inhibition diameters on *E. coli* agar plate.

| Hour | Peptide+Chl | | Peptide | PBS + chloramphenicol | PBS |
|---|---|---|---|---|---|
| 16th hour | 2% | 2.8 | ND | 2.9 | ND |
| | 3% | 2.7 | ND | 3.4 | ND |
| | 4% | 2.8 | ND | 3.1 | ND |
| | 5% | 2.9 | ND | 3.2 | ND |
| 40th hour | 2% | 2.1 | ND | 2.3 | ND |
| | 3% | 2.1 | ND | 2.4 | ND |
| | 4% | 2.4 | ND | 2.6 | ND |
| | 5% | 2.8 | ND | 3.1 | ND |

## 2.3 Fullerene-Peptide Conjugate

### 2.3.1 Synthesis of Fullerenol-Peptide Conjugate

#### 2.3.1.1 Synthesis of Fullerenol

After synthesizing fullerenol, IR spectrum analysis and solubility test were used for characterization. While fullerene is not soluble in water, fullerenol is. It was

observed that the product dissolve in water. Also, the peak between 3000-3500 $cm^{-1}$ indicates the hydroxyl groups on the fullerenol in Figure 52.

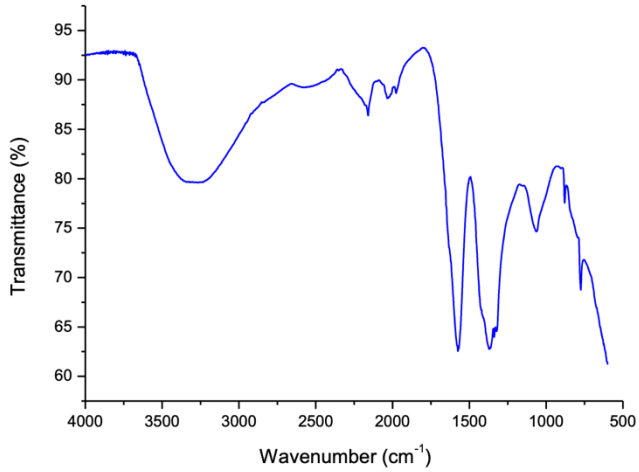


Figure 52: IR spectrum of fullerenol.

### 2.3.1.2 Synthesis of Activated Fullerenol

To characterize activated fullerenol, $^{1}$H-NMR was used. Hydrogen doublets in 7.0 and 8.1 ppm indicate the activation of fullerenol was completed and it is consistent with the literature data (Figure 53).[45]

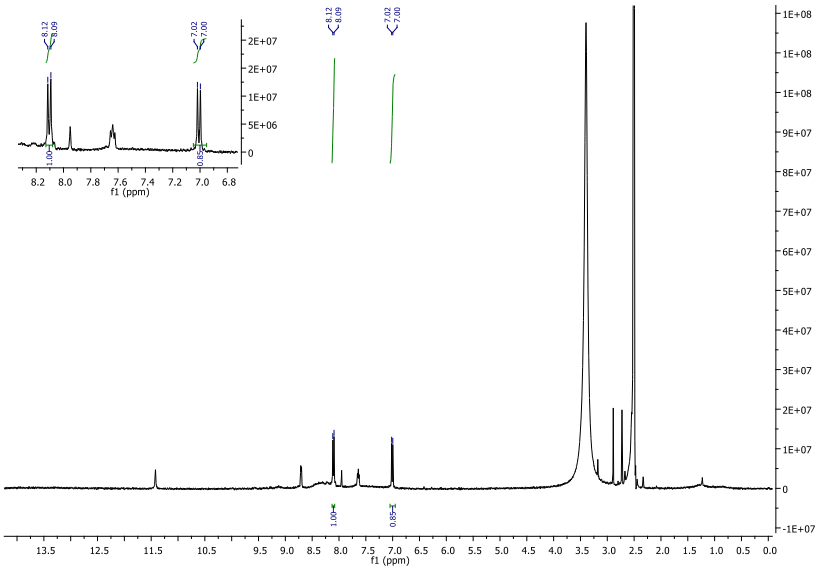


Figure 53: $^{1}$H-NMR spectrum of activated fullerenol in $d_6$-DMSO.

### 2.3.1.3 Attachment of Ac-IKYLSVN-$NH_2$ to Fullerenol

NMR spectrum of both Ac-IKYLSVN-$NH_2$ and Ac-IKYLSVN-$NH_2$-fullerenol conjugate were analyzed to observe whether the peptide was attached to fullerenol or not. In figures 54 and 55, $^1$H-NMR spectra of the peptide and fullerenol-peptide conjugate are depicted, respectively. When two $^1$H-NMR data were overlayed, it was seen that several peaks of Ac-IKYLSVN-$NH_2$-fullerenol conjugate were slightly shifted compared to the peaks of Ac-IKYLSVN-$NH_2$. Thus, it can be concluded that the peptide was bonded to fullerenol successfully.

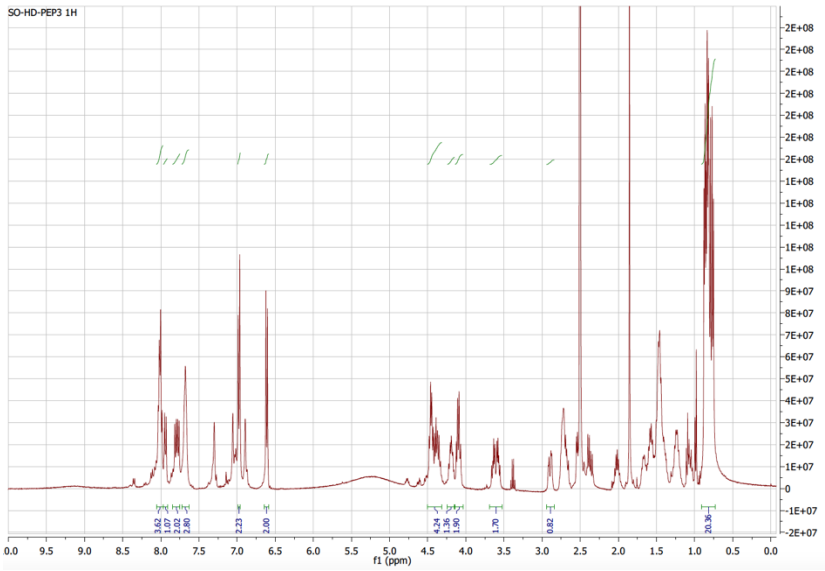


Figure 54: $^1$H-NMR spectrum of Ac-IKYLSVN-$NH_2$.

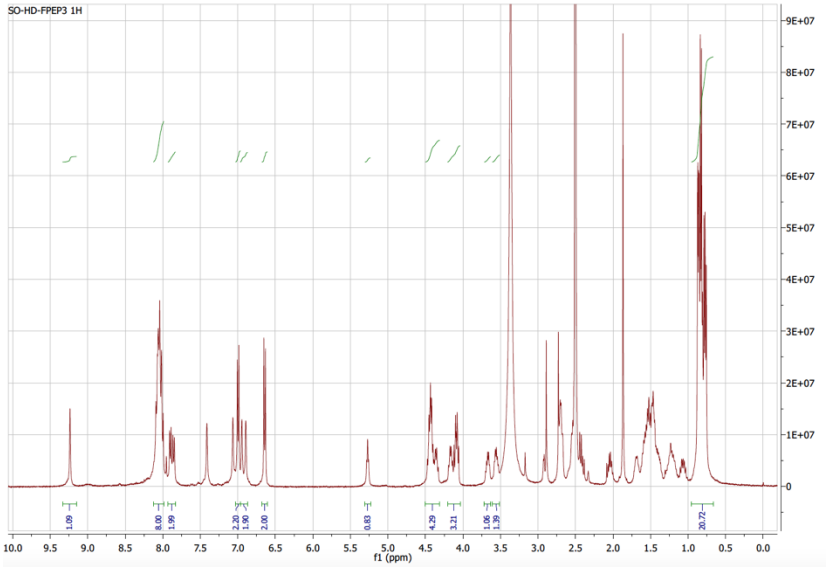


Figure 55: $^{1}$H-NMR spectrum of Ac-IKYLSVN-$NH_2$-fullerenol conjugate.

Also, the surface topography of fullerenol-peptide conjugate was examined by using Scanning Electron Microscope (SEM). In Figures 56 and 57, the topography can be seen in a scale of 10 µm & 4 µm and 10 µm & 2 µm, respectively.

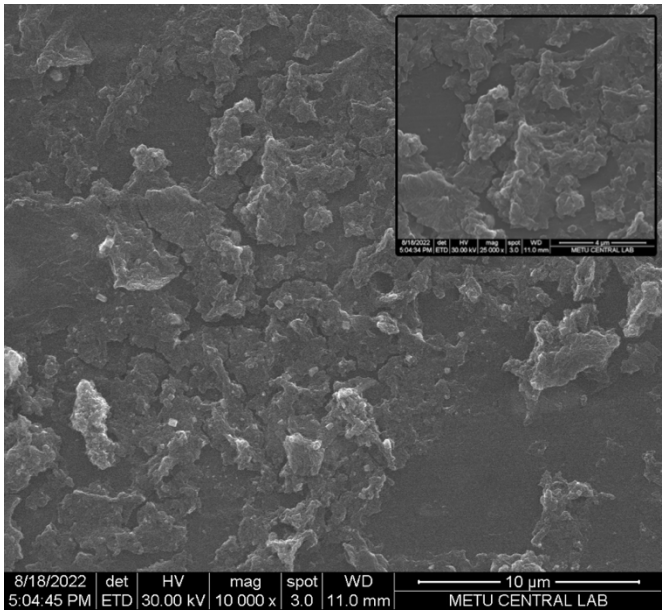


Figure 56: SEM image of the fullerenol-peptide conjugate in scales of 10 µm and 4 µm.

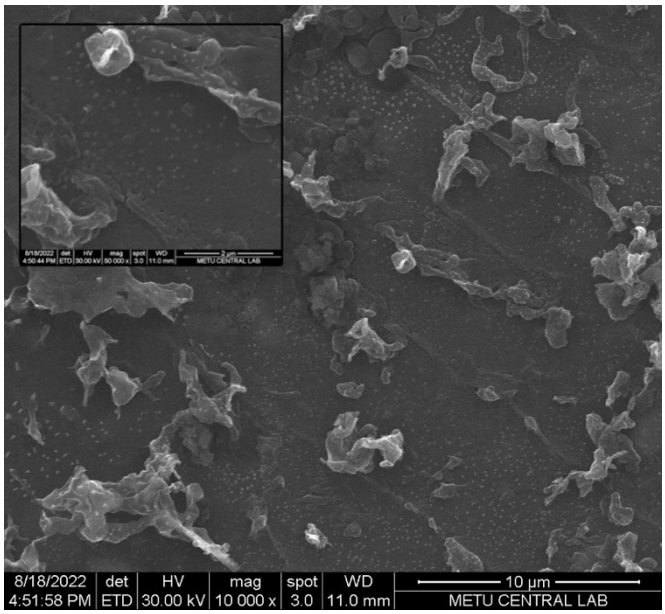


Figure 57: SEM image of the fullerenol-peptide conjugate in scales of 10 µm and 2 µm.

At last, HPLC analysis was conducted for Ac-IKYLSVN-$NH_2$-fullerenol conjugate. The retention time (RT) of the conjugate should be different from the peptide's RT if the peptide was successfully attached to fullerenol. The same method and column were used for the conjugate as Ac-IKYLSVN-$NH_2$ (Figure 58). RT of Ac-IKYLSVN-$NH_2$ is 14,34 while RT of the conjugate is 14,23 which was nearly the same as can be observed in Figure 59. Unfortunately, this states that the peptide couldn't be attached to fullerenol.

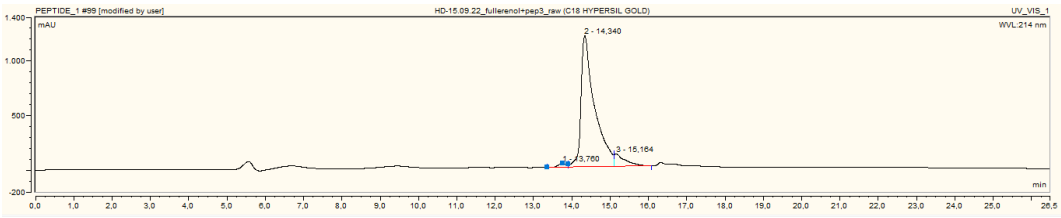


Figure 58: HPLC chromatogram of Ac-IKYLSVN-$NH_2$-fullerenol conjugate.

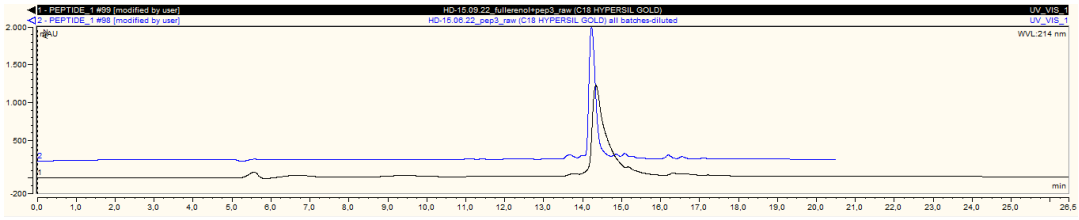


Figure 59: Overlay of Ac-IKYLSVN-$NH_2$-fullerenol conjugate and Ac-IKYLSVN-$NH_2$.

# CHAPTER 3

# CONCLUSION

In this study, Ac-$(FKFE)_2$, Ac-$(FKFE)_3$, and Ac-IKYLSVN-$NH_2$ peptides were synthesized and purified successfully. Mass analyses of the peptides were performed for characterization. Their gel formation was examined and found that all of them can form a hydrogel. However, Ac-$(FKFE)_2$ gel was not resistant to mechanical forces and the gel could not form when the structure is disturbed. Thus, biological studies were conducted for Ac-$(FKFE)_3$, and Ac-IKYLSVN-$NH_2$ peptides.

In order to perform biological tests on Ac-$(FKFE)_3$ gel, its pH was tried to be adjusted to ~7.3. It was expected that the gel and DMEM would be compatible for up to five days in an incubator or not. Unfortunately, the gel and DMEM in TCP wells were dried after two days in an incubator which makes further biological analysis impossible.

The same compatibility test was applied to Ac-IKYLSVN-$NH_2$ gel however the result was the same with Ac-$(FKFE)_3$ gel. Thus, other biological tests were planned for the peptide. Antibacterial susceptibility and drug release capability of the peptide were examined by agar plate and disc diffusion tests, respectively. The antibacterial effect was not observed in the agar plate test while the drug release capability of the peptide was observed with the disc diffusion test.

At last, a fullerenol-Ac-IKYLSVN-$NH_2$ conjugate was tried to be synthesized and characterized. However, HPLC analysis showed that the conjugation of peptide to fullerenol was failed. It must be repeated using a longer reaction time or higher stoichiometry of peptides.

# CHAPTER 4

# EXPERIMENTAL

## 4.1 Materials and Methods

### 4.1.1 Materials

Ultrapure water was provided by using the Milli-Q® Direct Water Purification System at the Department of Chemistry.

Commercial amino acids, HBTU, and Rink amide resin were acquired from Chem-Impex International Inc.

DMF, DCM, Diethyl Ether, and Acetonitrile were purchased from Carlo Erba.

DIEA which is used as an activator was supplied from Fisher Scientific.

THF and TIPS were purchased from Sigma Aldrich.

Acetic Acid and Pyridine were provided from Merck.

Agilent LC-MS was used for examining the peptides after the synthesis.

Eppendorf high-speed centrifuge was used to precipitate the peptides and peptide conjugate.

Telstar Cryodos branded freeze dryer was used to lyophilize the peptides.

Dionex Ultimate 3000 HPLC with DAD detector was used for purification and assay test of the peptides.

0.22 μm PTFE filters used for HPLC sample preparation were purchased form ISOLAB.

Kudos ultrasonic bath, Labnet VX100 vortex, Shimadzu AY220 analytical balance were used during both the synthesis and analysis of the peptides.

Bruker Spectrospin Advance DPX was used to obtain $^{1}$H-NMR spectrum of fullerenol-peptide conjugates.

PBS tablets were purchased from Sigma Aldrich.

### 4.1.2 Manual Microwave Peptide Synthesizer

CEM Discover® Bio Manual Microwave Peptide Synthesizer was used for peptide synthesis. The reaction is 5 to 10 times faster in the synthesizer, thus, larger batches can be synthesized in a very short period of time. Nitrogen gas, which was provided from Linde, was used for stirring by creating bubbles in the reaction vessel throughout the synthesis.

### 4.1.3 High Performance Liquid Chromatography

Dionex Ultimate 3000 Series HPLC with diode array detector was used for both purification and assay test of the synthesized peptides. 250x10mm 5µm Thermo Scientific ODS Hypersil™ semi-preparative HPLC column was used for purification of the Ac-$(FKFE)_2$ and Ac-$(FKFE)_3$ 250x10mm 5µm Thermo Scientific ODS Hypersil™ semi-preparative HPLC column was used for purification of the Ac-$(FKFE)_3$. 150x4.6mm 5µm, C18 Thermo Scientific ODS Hypersil™ HPLC column was used for assay test of Ac-$(FKFE)_2$ and Ac-$(FKFE)_3$. 150x4.6mm 5µm, C18 Thermo Scientific ODS Hypersil™ Gold HPLC column was used for assay test of the Ac-IKYLSVN-$NH_2$.

#### 4.1.3.1 Purification Method of Peptides

High pressure liquid chromatography is one of the most common purification methods along with techniques such as ion exchange, flash, or size chromatography.[46] Purification was performed for Ac-$(FKFE)_2$ and Ac-$(FKFE)_3$ since the purity of the peptide Ac-IKYLSVN-$NH_2$ was higher than 85%.

Dionex Ultimate 3000 Series HPLC with diode array detector which was set 210 nm wavelength and 250x10mm 5µm Thermo Scientific ODS Hypersil™ semi-preparative HPLC column were used for purification of both Ac-$(FKFE)_2$ and Ac-$(FKFE)_3$. The column compartment was set at 40°C and the flow rate was 3.000 mL/min. Gradient elution 0-50-90% was applied thus 2 mobile phases were prepared. Mobile phases A and B consist of Milli Q : TFA (1000:1) and ACN : TFA(1000:0.8), respectively. The run time was 150 minutes and 2 mL of the sample solution was injected.

Sample Preparation of Ac-$(FKFE)_2$

~26 mg of the crude peptide was solved with 0.5 mL Milli-Q, 0.7 mL ACN, and 0.3 mL HFP in an Eppendorf tube. The tube was placed in an ultrasonic bath until it dissolves completely which took ~45 minutes. Then the solution was filtered through 0.22 µm PTFE filter.

Sample Preparation of Ac-$(FKFE)_3$

~35 mg of the crude peptide was solved with 0.25 mL Milli-Q, 1 mL ACN, 0.75 mL Acetic Acid, and 0.5 mL HFP in an Eppendorf tube. The tube was placed in an ultrasonic bath until it dissolved completely which took ~45 minutes. Then the solution was filtered through 0.22 µm PTFE filter. To dissolve the peptide for HPLC sample preparation, the solvent (Milli-Q, ACN, acetic acid and HFP solution) can be prepared directly proportional to the amount of the crude peptide.

#### 4.1.3.2 Assay Test of Peptides

The widest range of methods for peptide assay tests has been developed in HPLC. Thus, it was used to quantify Ac-$(FKFE)_2$, Ac-$(FKFE)_3$, and Ac-IKYLSVN-$NH_2$. As well as the purification method described above, the same mobile phases (Milli Q : TFA (1000:1) and ACN : TFA(1000:0.8) and column compartment temperature (40 °C) were applied for all of the assay tests.

For Ac-$(FKFE)_2$ and Ac-$(FKFE)_3$, the run time was 25 minutes and 20 µL of the sample solution was injected. 150x4.6mm 5µm, C18 Thermo Scientific ODS Hypersil™ HPLC column was used and the flow rate was 0.500 mL/min. The concentration of the sample solution was ~1 mg/mL.

For Ac-IKYLSVN-$NH_2$, the run time was 35 minutes and 20 µL of the sample solution was injected. 150x4.6mm 5µm, C18 Thermo Scientific ODS Hypersil™ Gold HPLC column was used and the flow rate was 0.400 mL/min.

### 4.1.4 Nuclear Magnetic Resonance (NMR)

Bruker Spectro Spin Advance 400 was used to observe $^1$H-NMR data. To solve the fullerenol-peptide conjugate sample, $d_6$-DMSO was used.

### 4.1.5 Solid Phase Peptide Synthesis Protocol

Solid phase peptide synthesis basically consists of deprotection, coupling, and cleavage steps.[47] The steps were repeated until the desired peptide sequence was obtained. After the last coupling step, the resin was dried with DCM and the cleavage solution was transferred to the reaction vessel.

The synthesis steps below were described for 0.3 mmol. All peptide syntheses were conducted in the same way. Only their amount was changed based on the need.

#### 4.1.5.1 Solutions to be Prepared

Preparation of 0.6 M DIEA Solution

Transfer 5.0 mL of DIEA into a 50 mL volumetric flask and complete the volume with DMF.

Preparation of 20% Piperidine Solution

Transfer 20.0 mL of piperidine into a 100 mL volumetric flask and complete the volume with DMF to 100 mL

Preparation of $Ac_2O$ Solution

Mix 200 µL Acetic Anhydride, 200 µL Pyridine, and 5.0 mL DMF in a glass vial.

Preparation of Cleavage Solution

Transfer 500 µL TIPS and 500 µL Milli-Q into a 20 mL volumetric flask and complete the volume with TFA, carefully.

#### 4.1.5.2 Deprotection of the Resin and Amino Acid

906 mg Rink amide resin (substitution: 0.331 meq/g) which equals to 0.3 mmol was weighed in the reaction vessel. Then, 10 mL DMF was transferred into the vessel to swell the resin. After 30 minutes swelling was completed and DMF was drained. ~12 mL of 20% piperidine solution was transferred into the vessel and carefully stirred a couple of times and waited for 10 minutes at least. Then the solution was drained, and the resin was washed with DMF thoroughly. The step with the 20% piperidine solution was repeated to be sure of deprotecting all Fmoc-protected tips. Once more, washing with DMF was performed and then coupling steps were applied. After the completed coupling of the first amino acid to the resin, deprotection steps were repeated to couple another amino acid. All deprotection steps were the same for both the resin and the amino acids.

#### 4.1.5.3 Coupling

570 mg HBTU which equals 1.5 mmol was weighed in a test tube along with 1.65 mmol amino acid (5.5 fold) and to activate the C terminal of the amino acid, 6 mL 0.6 M DIEA solution was added. Vortex was used to completely dissolve both the HBTU and amino acid. After obtaining a clear solution, it was transferred to the vessel and waited for at least 50 minutes to the reaction takes place. Then the solution was drained, and the resin was washed with DMF thoroughly. If the sequence is long or the coupling of the amino acid is difficult, then the $Ac_2O$ solution can be used to cap the unreacted sites of the amino acids to prevent further impurities and improve the purity of the synthesized peptide.[48] Prepared $Ac_2O$ solution was transferred after washing the resin with DMF and waited for 10 minutes. At last, the resin was washed with DMF again. During both deprotection and coupling steps, the Kaiser test can be used to determine whether the Fmoc is deprotected or coupling is completed or not. After 20% piperidine solution was rinsed with DMF, a couple of resin beads were transferred to a test tube and Kaiser solutions were added. Later, the test tube was heated up to 110 °C and color change of the beads was observed. If the beads are colorless, the coupling is completed. However, if the beads have blue/purple color, then coupling is failed. When the Kaiser test was used to see whether the deprotection was completed or not, then blue/purple color should be observed.

#### 4.1.5.4 Cleavage

After the peptide sequence was synthesized (and if applicable acetylation of the sequence was completed), the resin was washed with DMF and DCM, respectively. It was dried with the vacuum pump for nearly 30 minutes. After the resin was fully dried, 20 mL cleavage solution was transferred to the reaction vessel and waited for 2 hours. At the same time, ~30 mL ether was placed in the freezer. Then cleaved peptide solution was dropped into a falcon tube which was filled with cooled down ether, then it was centrifuged. After that, the ether was poured down carefully and

fresh ether was added. The solution was centrifuged at least 4 times to precipitate the peptide completely. At last, the ether was poured down and the peptide in the tube was placed in the fume hood to dry.

### 4.1.6 Gelation Protocols

After completing the synthesis and mass analysis of the peptide the first step was to assess whether the peptide could form hydrogel or not for further processes. In literature Ac-$(FKFE)_2$, Ac-$(FKFE)_3$, and Ac-IKYLSVN-$NH_2$ have different routes.

#### 4.1.6.1 Ac-$(FKFE)_2$ Gelation Protocol

Minimum and maximum critical coagulation concentrations of Ac-$(FKFE)_2$ are 1 mM and 5 mM, respectively.[43] 5 mM NaCl stock solution was prepared by weighing 29.22 mg NaCl into a 100 mL volumetric flask and completing the volume with Milli Q. Between these concentrations, the peptide was expected to form a gel. Thus, 2.5 mM NaCl solution was prepared by transferring 12.5 mL of 5 mM NaCl stock solution into a 50 mL volumetric flask and completing the volume with Milli Q. To prepare the Ac-$(FKFE)_2$ based gel, 3 mg of Ac-$(FKFE)_2$ was weighed in a 1 mL Eppendorf tube and 300 μL of 0.5 mM NaCl solution was added. Then, the tube was placed in an ultrasonic bath for 5 minutes.

#### 4.1.6.2 Ac-$(FKFE)_3$ Gelation Protocol

Minimum and maximum critical coagulation concentrations of Ac-$(FKFE)_3$ are 0.1 mM and 1 mM, respectively.[43] 5 mM NaCl stock solution was prepared by weighing 29.22 mg NaCl into a 100 mL volumetric flask and completing the volume with Milli-Q. Between these concentrations, the peptide was expected to form a gel. Thus, 0.5 mM NaCl solution was prepared by transferring 5 mL of 5 mM NaCl stock solution into a 25 mL volumetric flask and completing the volume with Milli Q. To

prepare the Ac-$(FKFE)_3$ based gel, 3 mg of Ac-$(FKFE)_3$ was weighed in a 1 mL Eppendorf tube and 300 µL of 0.5 mM NaCl solution was added. Then, the tube was placed in an ultrasonic bath for 20 minutes.

#### 4.1.6.3 Ac-IKYLSVN-$NH_2$ Gelation Protocol

To prepare the Ac-IKYLSVN-$NH_2$ based gel, 3 mg of the peptide was weighed in a 1 mL Eppendorf tube and 300 µL of Milli Q was added.[17] Then, the tube was placed in an ultrasonic bath for 30 minutes.

### 4.1.7 Synthesis of Fullerenol-peptide Conjugates

To achieve fullerenol-peptide conjugates, first fullerenol and activated fullerenol were synthesized.

#### 4.1.7.1 Solutions to be Prepared

Preparation of NaOH Solution

20 mg NaOH was weighed in a 20 mL volumetric flask and the volume was completed with Milli-Q.

#### 4.1.7.2 Synthesis of Fullerenol

160 mg fullerene was weighed in a 200 mL round bottom flask and 100 mL toluene was added. 2 mL of NaOH solution was transferred into the flask and it was stirred at room temperature. Following 8-12 drops of 50% $H_2O_2$ solution, tetrabutylammonium hydroxide (TBAH) which is a phase transfer catalyst was added. When all solid components were dissolved, the flask was placed on a magnetic stirrer for 5 days. At the end of 5 days, the toluene layer was removed and fullerenol was precipitated by adding ethanol. The precipitated compound was

observed to be dark brown, and it was centrifuged 5 times by using ethanol. The compound can be characterized by its solubility in water.[45]

#### 4.1.7.3 Synthesis of Activated Fullerenol

120 mg fullerenol was weighed in a 100 mL round bottom flask and 15 mL DMF was added to form a suspension. Then flask was sonicated for one hour to obtain a homogeneous suspension. 800 mg *p*-nitrophenyl chloroformate, 4 mL anhydrous pyridine, and 40 mg DMAP were added at 0°C. The flask was placed on a magnetic stirrer for 72 hours under $N_2$ atmosphere.[45] The flask was sonicated for 1 hour every 8 hours. At the end of 48 hours, the solid compound was precipitated by centrifuging with diethyl ether. Following precipitation, the compound was washed with diethyl ether, DCM, and isopropyl alcohol, respectively to eliminate impurities.

#### 4.1.7.4 Attachment of Ac-IKYLSVN-$NH_2$ to Fullerenol

40 mg activated fullerenol was weighed in a 250 mL round bottom flask and ~100 mL DMF was added. Then, 100 mg Ac-IKYLSVN-$NH_2$ was added, and the flask was placed in an ultrasonic bath for 40 minutes.[45] When all solid components were dissolved, the flask was placed on a magnetic stirrer for 72 hours under $N_2$ atmosphere. The flask was sonicated for 1 hour every 12 hours. After completing the synthesis, ~10 mL of the product was transferred into a falcon tube and the tube was filled up to ~30 mL with cold ether. Then, the solution was centrifuged 5 times at 4500 RPM for 5 minutes.

To observe whether the conjugate was formed or not, NMR spectroscopy was used. ~15 mg Ac-IKYLSVN-$NH_2$ was weighed into an NMR tube and ~0.4 mL $d_6$-DMSO was added. Also, ~10 mg conjugate was weighed into an NMR tube and ~0.4 mL $d_6$-DMSO was added. Both tubes were placed in the ultrasonic bath for ~30 minutes.

### 4.1.8 Biological Studies

Antibacterial susceptibility of Ac-IKYLSVN-$NH_2$ and drug release capability of its gel were analyzed in Assoc. Prof. Dr. Batur Ercan's research laboratory by Yiğithan Tufan and Cemre Örsel.

#### 4.1.8.1 Solutions to be Prepared

Preparation of PBS Solution

1 PBS tablet was solved in 200 mL Milli-Q.

Preparation of Chloramphenicol Stock Solution

40 mg chloramphenicol was weighed in a 2 mL Eppendorf tube and 1 mL ethanol was added then the tube was placed in an ultrasonic bath for ~2 minutes.

Preparation of PBS + Chloramphenicol Solution

10 µL chloramphenicol stock solution was transferred to a 10 mL volumetric flask and the volume was completed with PBS solution.

#### 4.1.8.2 Gelation Protocol

To prepare the 2% Ac-IKYLSVN-$NH_2$ based gel, 8 mg of the peptide was weighed in a 1 mL Eppendorf tube, and 400 µL of PBS + chloramphenicol solution was added. Then, the tube was placed in an ultrasonic bath for 10 minutes. If the peptide does not dissolve in 10 minutes, then the tube can be placed in a vortex for ~2 minutes followed by a 5-minute ultrasonic bath.

#### 4.1.8.3 Kirby Bauer Disc Diffusion Test

To evaluate the drug release capability of Ac-IKYLSVN-$NH_2$ gel, a disc diffusion test was conducted. Chloramphenicol was used as an antibiotic agent against S. *aureus* (ATCC 25923) and *E. coli* (ATCC 10536) bacteria strains. For S. *aureus* and *E. coli,* Tryptic Soy Broth (TSB) and Luria Bertani Broth (LBB) were used as mediums, respectively. For LB agar, 15 g/L agar and 25 g/L LBB solutions were mixed and autoclaved for 20 minutes at 121 ºC. The same procedure was applied for TSB agar with 15 g/L agar and 30 g/L TSB solutions. By autoclaving the mixtures, the agar was dissolved, and the mixtures were sterilized. Sterilized solutions were poured down to petri dishes and placed in a 4 ºC refrigerator for 24 hours.

S. *aureus* and *E. coli* were proliferated for 18 hours in Tryptic Soy agar and Luria Bertani agar, respectively. Then, S. *aureus* was transferred in 3% TSB medium and *E. coli* was transferred in 2.5% LB medium. Both bacteria strains were grown in 37 ºC 200 RPM shaking for 18 hours until they reach the saturation.

After the bacteria strains reach the saturation, both were diluted with PBS to have an optical density of 0.1 in each 600 nm which equals $10^8$ colony forming unit (CFU) in 1 mL. To reach the aimed seeding density ($10^6$), 1:100 dilution was performed with PBS, again.

On each Agar plate, 4 wells were opened by using a 2 mm punch. All of the wells were filled up to their full volume by spatula with 2 gel samples, positive and negative controls. 2%, 3%, 4%, and 5% Ac-IKYLSVN-$NH_2$ gel samples were prepared with PBS and PBS + chloramphenicol solutions. PBS was used as the negative control while PBS + chloramphenicol solution was used as the positive.

500 µL bacteria suspension was spread on the agar plates with a cell spreader. Then wells were filled up to the point they were full of defined samples and control solutions. The agar plates were placed in the incubator for 24 hours. After 24 hours, the zone of inhibition of each sample was measured.

#### 4.1.8.4 Antibacterial Testing of Ac-IKYLSVN-$NH_2$

The same agar plate preparation procedure in title 4.1.8.3 was followed for S. *aureus* and *E. coli* for antibacterial testing of Ac-IKYLSVN-$NH_2$. Then two solutions of the peptide were prepared with concentrations of 5 mg/mL and 10 mg/mL. 10 µL of each solution was dripped on 4 areas of the agar plates. Then the plates were placed in an incubator for 24 hours and the zone of inhibition of each sample was measured.

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