SYNTHESIS AND BIOPHYSICAL CHARACTERIZATION OF IMIDAZOLE AND DEHYDROPEPTIDE BASED SELF-ASSEMBLED NANOSTRUCTURES

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OBJECTIVE

- Synthesis of small dehydro-peptide based self-assembled peptides having different chemical structure.
- Purification of these peptides using HPLC.
- Bio-physical characterization of synthesized peptides using different techniques such as DLS, UV-visible, NMR, FT-IR, MASS and TEM.
- Studied Anti-microbial activities of these synthesized peptides.

ABSTRACT

The study focuses on the synthesis and characterization of imidazole-containing dehydropeptide nanoparticles through solution and solid-phase peptide synthesis. In pharmaceutical chemistry, imidazole and its derivatives are among the most significant and often utilized heterocycles. Due to the distinct structural characteristics of the imidazole ring, imidazole derivatives can bind to a variety of enzymes and receptors with ease through a variety of weak interactions, exhibiting a broad spectrum of biological and pharmacological effects. Many imidazole derivatives have been utilized extensively as anti-inflammatory, anticancer, antiviral, antibacterial, and other therapeutic agents in the field of medicinal chemistry. Therefore, in this study, the synthesized nanostructures will be evaluated for the anti-microbial properties in Gram-positive and Gram-negative bacteria through Zone of Inhibition (ZOI) and Minimum Inhibitory Concentration (MIC). The physical characterization of the subjected nanoparticles was done using various spectroscopic techniques, including Infrared (IR), Nuclear Magnetic Resonance (NMR), and Ultraviolet (UV) spectroscopy, and size and zeta-potential analysis of the nanoparticles were carried out by DLS. It was observed that the synthesized peptide was self-assembled in aqueous media in the range of 222-650 nm with a zeta potential ranging from (+4) to (+9). Furthermore, it was observed that the projected nanostructures showcased initial antimicrobial activity, thereby contributing to potential applications in the field of nanomedicine.

Keywords:

Dehydro-peptide, MIC, Anti-microbial activity, DLS, HPLC, TEM, FT-IR.

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LIST OF ABBREVIATIONS AND SYMBOLS:

Fmoc	-	Fluorenylmethoxycarbonyl protecting group
Boc	-	Di-tert-butyl pyro carbonate
Phe	-	Phenylalanine
ΔPhe	-	α, β Dehydro phenylalanine
TFA	-	Trifluoro acetic acid
DCM	-	Di-Chloromethane
DMF	-	Di-methyl formamide
CTC-Resin	-	2-chlorotrityl chloride
DLS	-	Dynamic Light Scattering
TEM	-	Transmission Electron Microscopy
NMR	-	Nucleus Magnetic Resonance
FT-IR	-	Fourier transform infrared
ZOI	-	Zone of inhibition
MIC	-	Minimum inhibitory concentration
mg	-	milligram
µg	-	microgram
min	-	minute
ml	-	millilitre
mM	-	millimolar
rpm	-	revolutions per minute

Chapter 1

Introduction

Self-assembly of amino acid-based peptides into a broad range of nanostructures, the majority of which are biodegradable and have significant biological activities[1]. The synthetic techniques, distinct structural features, and potential biological applications of self-assembled peptides as biomaterials for disease theranostics have been extensively and thoroughly investigated in recent decades. Peptide-based materials have applications in bioelectronics, imaging techniques, solar energy transformation, drug delivery, tissue engineering, peptide vaccinations, antimicrobial and anti-cancer therapy[2].

There are various reasons why the peptide-based nanomaterial is attractive[1]

1) Easy synthesis permits molecular modification and cost effective.

2) Biodegradable and biocompatible.

3) Control secondary structure like, α-helices and β-sheets, possibly altered for enhancing their self-assembly mechanism.

Peptides that form β-sheets exhibit a remarkable capacity for assembly into one-dimensional (1D) nanostructures via intramolecular hydrogen bonding[3]. Interactions between one-dimensional (1D) peptide-based nanostructures can give rise to three-dimensional (3D) networks. On certain secondary structures, peptides offers special flatform to synthesize materials having tunable structural features at the nanoscale. Bioactive hydrogels that mirror the content and characteristics of natural extracellular matrix (ECM) can be made by adding certain amino acid sequences or modifying their self-assembled structures, which further adjusts the biodegradability.
Molecular self-assembly is an exciting approach for generating nanoscale materials because of its simplicity of use[4][5][6]. Molecules spontaneously arranged themselves into ordered structures at various length scales by this free energy driven mechanism. Self-assembled nanostructures, such as nanofibers, nanotubes, vesicles and micelles are built by synthetic molecules like amino acids, oligo- and polypeptides, polymers, dendrimers, and π-conjugated polypeptides[7]. Main advantage of using this technique to create nanoscale objects is that the structural characteristics of final assemblies can be easily and precisely adjusted based on molecular chemistry, assembling environment (temperature, solvents, pH and co-assembling molecules), and assembly kinetics. When using such self-assembling substances, main objective involves fulfilling their intended purpose rather than necessarily creating thermodynamically stable structures. Thus, structures that are metastable, or kinetically trapped, may increase the diversity of these materials by providing structures that change over time. For examples, artificial cell scaffold materials should support and signal native or transplanted cells before breaking down when they are no longer required. Self-assembling networks termed as hydrogels that can break down over a specific time frame, have considerable potential for transportation of cells in order achieve maximal performance in a less invasive manner.

Despite having several biological applications, the major drawback of self-assembled peptide(SAP) is its limited proteolytic stability. To overcome this drawback, certain strategies are used like introducing double bond in peptide structure, using D-amino acids instead of L-amino acid, incorporation of un-natural chemically modified amino-acid, introducing cyclization, N- and C-terminal modification like N-terminal acetylation or C- terminal amidation, these alterations can improve the proteolytic stability of peptides while preserving their overall structure and function[8][9][10].

This present research thesis work involves, preparation and biophysical-characterization of imidazole containing dehydro-peptide, Ac-ΔPhe-Pro-βAla-His-NH_2(**I**), Ac-ΔPhe-Pro-Arg-βAla-His-OH(**II**) and Ac-ΔPhe-Pro-Arg-βAla-His-NH_2 (**III**) having α, β- dehydro-phenylalanine (ΔPhe) residue. Addition of ΔPhe, an unsaturated homologue of the naturally occurring Phe having a double bond between its α and β carbon atoms, giving more proteolytic stability to the peptide and inducing more conformational restrictions in its backbone. Self-assembled nanostructures characterization was carried out by Dynamic Light Scattering (DLS), UV, FT-IR, NMR, Mass spectroscopy. Their shape and size also studied by TEM spectroscopy. This comprehensive analysis aids in understanding conformation with characteristics of these nanoparticles, that is crucial because of exploring their possible bio-medical functions, novel self-assembled nanostructures could thus have potential application to show the initial results of antimicrobial activity.

Chapter 2

Literature Review

Nanoparticles with dimensions ranging from 10 to 1000 nm have lately been recognized of potential colloidal drug carriers [3]. In comparison to their bulk materials, nanoparticles have a variety of different chemical and physical properties because of their enormous reactive and exposed surface area, as well as the quantum size effect resulting from particular electronic structures [11]. With their improved solubility and increased bioavailability, nanoparticles can target specific drug locations in the body and lengthen the duration the body resists a certain stimulus. These carriers shows safe delivery of hazardous therapeutic drugs, protection of non-target tissues and cells from side effects, and a reduction in drug amount and toxicity [12]. Conjugation of the drug to carrier and the targeting strategy are crucial for targeted therapy. Controlled drug release can be accomplished by adjusting physiological variables as pH, temperature, osmolality, or enzyme activity[13].

2.1 AMINO ACIDS AND PEPTIDES

According to Mehta and Mehta (2012), the general formula for representing an amino acid is RCH(NH2) COOH. Carboxylic acids containing an amino group are referred to termed amino acids. Amino acids with α structure are most abundant in nature. An amino (NH2) group, a carboxylic group, a hydrogen atom bonded to carbon, and a side chain are generally present in all amino acids[14].

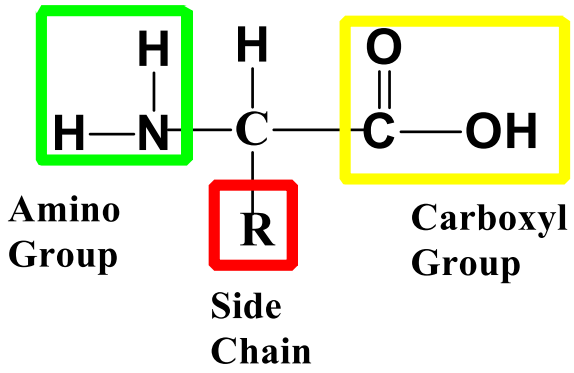


Figure 2.1: Amino Acid structure (www.education-portal.com)

Side chains of amino acids vary from one another. They possess distinctive characteristics properties due to the variation in side chain. Amino acids produce polymers referred to peptides and proteins. Peptides are short sequences of monomers of amino acids joined by peptide (amide) bonds. The following categories can be applied to peptides based on what number of amino acid units they contain:

- Oligopeptides, which consist of two to nine amino acid units.
- A polypeptide made up of 10–100 amino acid units.
- Protein represents a macro peptide with more than 100 amino acids.

A dipeptide is a peptide that contains two amino acids. In the same manner, a peptide having

three amino acid units is often referred to as a tripeptide, a tetrapeptide with four amino acid units, and furthermore. Hydrogen ions from the carboxyl end, hydroxyl ions from the amine end, or both, are released during the creation of each amide bond, which is why amino acids that have been integrated into peptides are referred to as "residue" (Mehta and Mehta, 2012)[14].

D/L-Amino Acids

One of the key characteristics that is essential for amino acids is their optical properties. Figure 2.2 displays the two enantiomeric configurations (L and D) for every amino acid, except for Gly. There is an increasing amount of interest in the use of synthesis of self-assembling peptide motifs with D- amino acids [15].

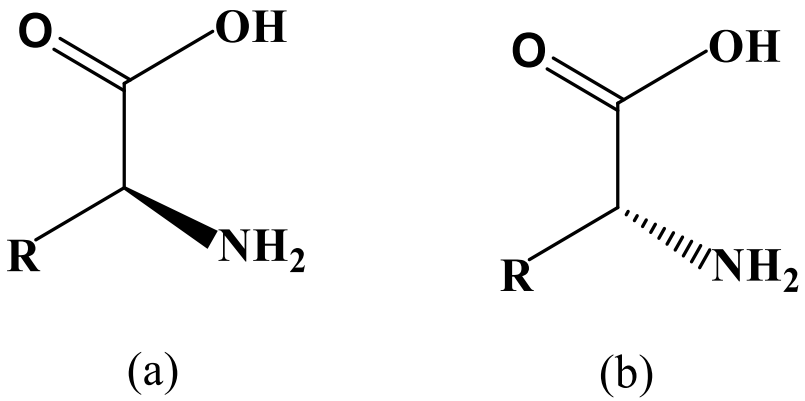


Figure 2.2:(a) Structure of an L-amino acid. (b) Structure of a D-amino acid.

2.2 MOLECULAR SELF-ASSEMBLY PROCESS

Specifically, and locally interacting molecules under thermodynamic and kinetic conditions give rise to ordered structures in a spontaneous process known as molecular self-assembly[16][17]. A well-defined nanostructure is dependent on the identification of particular molecules, which is brought about by interactions (non-covalent) like π-π stacking, hydrogen bonding, chiral dipole-dipole interactions, hydrophobic forces, and exchange-bonding. Despite their relative weakness when acting alone, these forces together have the ability to control how molecules self-assemble into ordered nanostructures.

In biological systems, self-assembly is play a crucial role, either to achieve biological functions or as part of pathogenic processes. Examples of this include the formation of self-assembly of phospholipid into biological membranes, the formation of secondary structures of DNA double helices and amyloid fibrils formation. Numerous biomimetic and biological materials are created by the "bottom-up" process of molecular self-assembly [18][19].

2.2.1 SELF ASSEMBLY IN PEPTIDES

Nucleic acids, proteins, peptides, and lipids are among the many biomolecules that are synthesized to form nanostructures. Because of their biocompatibility, capacity for particular molecular recognition, ease of modification, and accessibility for fabrication, supramolecular architectures made of biomolecules have a number of benefits [19][20].

Peptide-based biomaterial production is essentially inspired by natural systems, such as ability of self-assembly of cellular polypeptides/proteins into highly ordered fibrils in neurodegenerative diseases like Parkinson's and Alzheimer's[21]. Due to their inherent biological origin, peptides, a class of versatile different amino acids, are mostly used biomolecules for the formation of nanostructures [20][22]. Dipeptides, copolypeptides, dendritic peptides, amphiphilic peptides, bola-amphiphilic peptides, cyclic peptides, and surfactant-like The building blocks of peptides, known as cyclic, dendritic, copolypeptide, surfactant-like oligopeptide,amphiphile and aromatic peptides, were created and engineered for supramolecular creation structures as well as the investigation of their potential uses in biology and nanotechnology[23].

Dipeptides :

With the aid of multiple non-covalent interactions, dipeptides are the most basic building blocks capable of assembling themselves. A standard procedure can be used to synthesize them using at ease. As the smallest homoaromatic dipeptide derived from the Alzheimer's β-amyloid peptide Aβ-42[24]. The most common dipeptide is FF (diphenylalanine). Many different types of nanostructures, including nanotubes, nanoparticles, nanocrystals, etc., have been created using FF peptide[25][26].

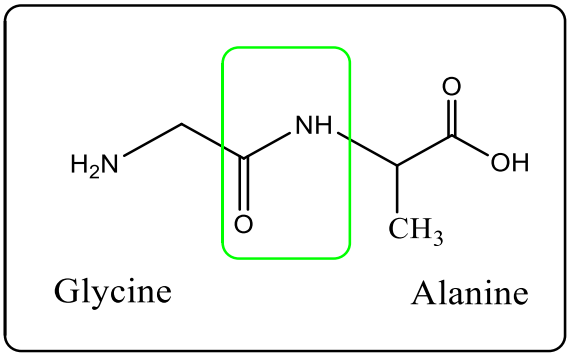


Figure 2.3: Dipeptide (Gly-Ala-OH)

Amphiphilic Peptides :

Amphiphilic peptides are characterized by charged hydrophilic head groups and hydrophobic tails A variety sel-assembled nanostructures with various structures like, nanotubules, nanofibres, and nanovesicles, peptide amphiphiles' hydrophobic alkyl chain essentially interacts with the hydrophilic group through electrostatic, hydrophobic, H-bonding and $\pi -\pi$ stacking. Hydrophilic part and the hydrophobic tails of the overall structures fold themselves in an aqueous environment to make them soluble in water [27]. It has been shown that under pressure, a C16 alkyl tail containing a V3A3E3 peptide can gel through a variety of electrostatic interactions. This gel has served as a scaffold in three-dimensional mesenchymal stem cell culture experiments[28][29].

Cyclic peptides :

A ring-shaped morphology or structure characterizes cyclic peptide chains, which are polypeptide chains. A variety of stable bonds, including amide, lactone, disulfide, ether, and others, can be used to link the ends of peptides to form rings. Many physiologically active cyclic peptides are formed through the process of N-to-C by formation of an amide bond between amino and carboxyl termini. Various naturally self-assembled c-peptides are finding important use in the medical realm [30].

Cyclic peptides can self-assemble into cylindrical nanostructures[31], which take on the appearance of tubular structures with a β-sheet due to intermolecular hydrogen bonding between amino acids. Because the side chains of cyclic peptides are often orientated on the exterior of the tube, their peptide backbone makes them particularly well-suited to produce nanotubes. An excellent choice for the creation of nanotubes is cyclic peptides since their peptide backbone is usually orientated so that the side chains are on the outside of the tube. The appropriate choice of peptide lengths and side chains of amino acids can control the cylinder's inner bore and outer facet properties[32].

A class of amphiphilic self-assembled peptides, which includes EAK, RDA-16 and RADA-16. Each of these peptides share a unique architectural structures negative and positive charged with hydrophobic side chains on the other have β-sheets. On the base of identification of a repeating 16-residue peptide motif with 16-residue EAK16-II, these peptides self-assembled into nanofiber scaffolds in yeast that may find use in 3D tissue cultures[20]. Surfactant-like oligopeptides have been generated with hydrophobic tails and hydrophilic heads by illustrating ideas from the self-assembly of lipids. Hydrophobic tails possess the ability to self-assemble into lipid molecules when they group together in water [33].

Certain peptide building blocks self-assembly obtained by means of pathogenic processes. ne, A primary recognition motif, Phe-Phe, is a well-known example of molecular self-assembly in Alzheimer's β-amyloid polypeptide[34]. Phe-Phe based building blocks have a wide range of applications and self-assembled into numerous functional nanostructures[23]. With an objective of enhancing the qualities and functionalities of FF building blocks, this field is expanding quickly.

2.3 SELF ASSEMBLED NANOSRTUCTURES

2.3.1 Nanofibrils

Natural or artificial peptides self-assembly of nanofibrils, which have a various uses in bionanotechnology. This fibrillization is pertinent to a number of diseases which like Alzheimer's, prion disorder, Parkinson's, Huntington's, and type 2 diabetes, where amyloid fibrils are deposited in numerous tissues and organs [35][36]. The β-amyloid polypeptide involves Phe-Phe in a significant way. The FF aromatic residue π stacking interaction is what results in this process take place [35].

When diphenylalanine is shielded by 9-fluorenylmethoxycarbonyl (Fmoc), it self-assembles into nanofibrils in water and forms hydrogel because of hydrogen bonding and π-π stacking [37][38]. For some applications, like cell culture with controlled drug, Fmoc-FF hydrogels are more advantageous because of the following characteristics:

- More rigid and strong
- Stable under a range of pH and temperature conditions
- Able to maintain itself. These exhibit rheological characteristics and a storage modulus (G).
  A molecular model that suggests the peptides formed a cylindrical structure with antiparallel β-sheet alignment and lateral π-π interactions between adjacent sheets has been proposed recently[39].

Molecular self-assembly for hydrogelation, that typically occurs in vivo or in vitro, is regulated or controlled by variety of natural enzymes, including phosphatase, β-lactamase, thermolysin, and phosphatase/kinase. There are two ways that hydrogels can be formed with enzyme tuning:

- Hydrogelators are created by removing inhibiting groups from precursor molecules, which allow the hydrogelators to self-assembles[22][40].
- Catalyzing the hydrogelator's bond formation.

For instance, according to **Ulijn et al.**, thermolysin catalyzes the protected Phe-Phe with Fmoc to create a hydrogel by self-assembly into three-dimensional fibril structures [41][42]. Enzymatic series of -controled self-assembly of nanostructures dealing with the Phe-Phe motif have been gentated [43][44]. Enzyme catalyzes the hydrolysis of β-lactam antibiotics. Through the use of the cephem nucleus as a linker, a hydrophilic group and a hydrogelator containing the Phe-Phe motif were joined to create a water-soluble precursor.

Chloroform and aromatic solvents are among the solvents that single dipeptide FF effectively gelates as Low Molecule-Mass Organogelators (LMOG)[45]. These thermo-reversible FF organogels have an apparent sol-gel transition temperature and are made up of long, entangled fibrils. The ageing process or external induction can transform a gel from a kinetically trapped state to a more thermodynamically stable crystal. It is easy to realize the structural transition of mesostable FF organogels into microcrystals by adding ethanol as a co-solvent to toluene[46].Several spectroscopy like, FTIR, CD, TGA, fluorescence spectroscopy, and XRD, are used to determine the self-assembly of aromatic peptide, Phe-Phe in hydrogels and

nanocrystals as a result of intramolecular interaction. Properties of the solvent, such as polarity and hydrogen bonding capacity, affect the regulation of assembly of organogold and control of nanostructures, microcrystals, and nano-fibers.

2.3.2 Nanowires

The carbon disulfide (CS2) FF dipeptides have the ability to self-assemble into 1D stiff nanowires[47]. A potential application for liquid nanowires produced by FF self-assembly is in nanopatterning, which involves combining materials to create nanocomposites with reinforcement [47]. When nanowires of FF vertically aligned on a substrate with solid-phase self-assembly, a peptide film of amorphous serves as a precursor [48]. Mass transport and surface nucleation regulate the growth on solid surfaces of nanowire during water vapour method self-assembly. Heat-responsive nanowire introduction can occur during the phase transition of FF molecular arrays[49].

2.3.3 Transition in nanotubes

The production of tubular nanostructures using proteins and peptides is a highly appealing option for possible building blocks, offering a multitude of applications. The most basic peptide, diphenylalanine (FF), may self-assemble into lengthy (~100 μm) tubular structures through aromatic residue stacking π-π stacking and hydrogen bonding. When a thiol group is added to an FF peptide, the resulting peptide's self-assembling capabilities can be changed. AFM study of the dried FF nanotubes (FNTs) revealed that they exhibited remarkable thermal durability up to 100ºC, but that they lose their structural integrity above that point. Because of their special qualities and stiffness, FNTs are the stiffest biological materials that can be used to create biocompatible nanodevices.

The production of nanotubes is the result of FF monomers first forming a 2D layer and then allowing this layer to close. There are numerous methods for aligning self-assembed peptides. When Phe-Phe in HFIP (1,1,1,3,3,3,3 hexa-fluoro-2-propanol) is distributed on salinized glass substrate, axial unidirectional growth of nanotubes exhibited array of FNTs with vertically aligned, and with the quick evaporation of HFIP and made up of nanotube arrays can be created with a thin film [50].

For FNTs to align horizontally, they must first be coated with magnetic nanoparticles on their surface and then exposed to an external magnetic field. Whereas FF plays a significant role in this context, information about the aromatic rings orientation can be obtained by measuring the nanotubes' alignment direction in the magnetic field. Multi-array sensors and nanodevices might be made with these FNTs[34].

FF has been molded into well-organized films on a variety of substrates (SiO2, Au, Pd, alumina, mica, quartz, and InP) by self-assembly of FF in an appropriate solvent, such as N-methyl-2-pyrolidone[51].

The cationic dipeptide at a physiological pH generated from FF (H-Phe-Phe-NH.HCI) can self-assemble into nanotubes[52][53]. A building block's hydrophilic or hydrophobic

characteristics and architecture remain constant, but by adjusting pH, temperature, solvent type and quality, concentration of the building molecule, and other factors, a varied morphology can be created[54]. Peptide building block concentration causes reversible transitions, such as those between CDNTPs and vesicle-like forms.Higher concentrations allow for the creation of tubular nanostructures through intramolecular interaction, which provides enough energy for association. Lower concentrations cause a reduction in free energy and the creation of vesicle-like structures through molecular rearrangement[55].

2.4 PEPTIDE-INORGANIC HYBRIDS

To improve properties and functions of nano biomaterials is based on combining biological or biomimetic building blocks with inorganic functional materials (polyoxometalates, nanoparticles, and nanocrystals) for applications in catalytic properties, photonic and electronic properties. Synthesis of such multifunctional hybrids involves the mixing of functional inorganic elements with FF dipeptide[45][56][57] and the nanotubes photoluminescent having lanthanide ions incorporated[58]. Water-dispersible colloidal spheres were produced by Phe-Phe containing organogels nanocrystals[49]. Water phase and an organogel phase containing nanocrystals are transferred during this procedure. Cationic dipeptide is protonated, this process is occur due to hydrophobic attraction and electrostatic interaction. Within this process, peptide encapsulated clusters (PECs) self-assemble to produce hybrid spheres through electrostatic contacts at first, and PEC stacking occurs through non-covalent interactions at a later stage[58] In addition to macromolecular polymers like FITC labelled dextran and fluorescein isothiocyanate (FITC), which are water-soluble small molecules including neutral, this hybrid supramolecular network can encapsulate a variety of materials[57]. Additionally, the hybrid supramolecular network forms core-shell nanostructures, where a hydrophobic drug self-assembles on the surface to create the drug's core.

The integration of self-assembled peptide with Lanthanide ions is also possible, and these ions possess optical properties that have wide-ranging uses in medical diagnosis, bio imaging, optical fibers, lasers, and lighting devices[59]. Park et al. produced PL peptide by incorporating lanthanides locally in the self-assembly of Phe-Phe nanotubes, where FF served as an antenna and matrix for the inclusion of lanthanide ions[60]. Through this method, self-assembly gains new capabilities with a variety of applications in electronics and optics

2.5 PEPTIDE SYNTHESIS

Amide bonds, commonly referred to as peptide bonds, are formed in the synthesis of poly peptide by coupling of various amino acids. This involves coupling the amine group (N-terminus) of one amino acid to the carboxylic acid group (C-terminus) of another amino acid. Theodor Curtis utilized the azide-coupling technique to create benzoylglycyl-glycine, the first N-protected dipeptide, in 1881. Emil Fischer produced unprotected glycylglycine in 1901 and described the synthetic dipeptide. The most widely used amino protecting group is Boc (tert-butyloxycarbonyl), which was first used by Carpino, McKay, and Albertson in 1957. Another scientist introduced fluorenylmethyloxycarbonyl (Fmoc), which is another amino protecting group. Methyl, ethyl and benzyl ester are commonly used for protection of carboxy group of amino acids. There are two processes for synthesising peptides: Solid Phase Peptide Synthesis (SPPS) and Solution Phase Peptide Synthesis (SPS).

2.5.1 Solid Phase Peptide Synthesis

Recognized technique for synthesizing peptides and proteins on solid support is called Solid Phase Peptide Synthesis (SPPS), and it was initially demonstrated by Robert Bruce Merrifield. In this method, resins were used as solid support which undergoes deprotection and then activated by protected amino acid for coupling followed by capping, this cycle continues and in end cleavage was done to get desired peptide sequence. A reagent such as trifluoroacetic acid or anhydrous hydrogen fluoride can cleave the peptide that is covalently bonded to the resin. The specific peptide sequence to be produced determines which resins are needed; for example, rink-amide resin is needed for the M-NH2 peptide sequence, whereas CTC resin is needed for the M-OH peptide sequence. Filtration can be used to hold onto the immobilized peptide on the beads. Reagents for the liquid phase and byproducts are eliminated through flushing.

Based on the successive steps of coupling-wash-deprotection-wash, solid phase peptide synthesis takes place out (Figure 2.3). Alpha amino group and first amino acid with transient protective groups on the reactive side chain are coupled to the solid support via the C-terminus. The protective group is eliminated and the resin is cleaned before adding another amino acid. Until the sequence is completed and the necessary peptide is released from the resin, the process is repeated. Solid phase peptide synthesis is constrained by its smaller scale and lower yield.

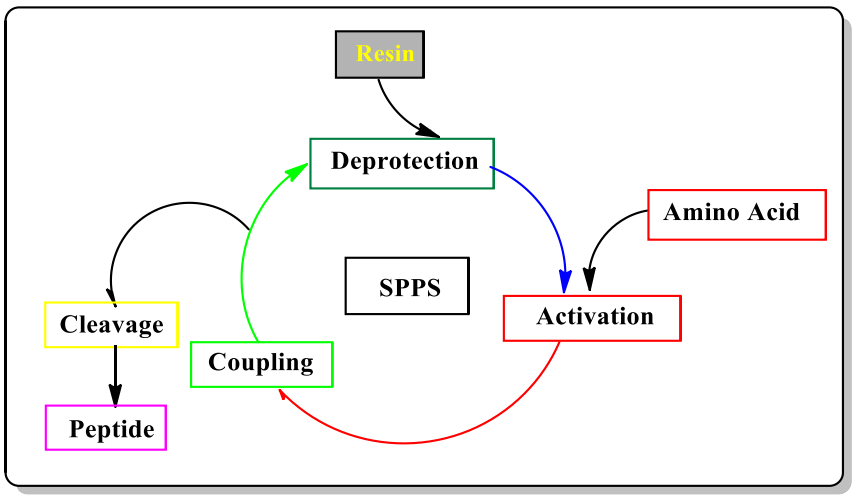


Figure 2.4:Solid Phase peptide Synthesis (moulder.temple.edu)

2.5.2 Solution Phase Peptide Synthesis

This method of peptide synthesis is traditional. Reactions are conducted in the solution phase using this approach. The peptide is separated and refined following the coupling of each amino acid, and then the subsequent amino acid is added. This approach has the benefit of making it simple to purify and characterize the intermediate following each stage in the formation of a peptide bond. To obtain the final, desired peptide in a high purity, the end products can be deprotected, purified, and described. This technique is simple and low-cost. Because the hydrophobic peptides can form intermolecular aggregates instead of interacting with organic solvents, their poor solubility is the main drawback of this approach. Thus, it is necessary to

select solvents with high solubility during the synthesis process. For this purpose, THF and DMF are commonly used as solvents for coupling reactions.

2.6 PEPTIDE PROTECTING GROUP

Given that amino acids are made up of basic and acid groups, polymerization of amino acids in reactions is possible because none of the amino acids are protected. Protective groups are utilized to stop this polymerization. There are now two protective groups that are frequently used: FMOC and BOC in their full forms.

2.6.1 Boc Protecting Group

N-tert-butoxycarbonyl, or t-BOC derivatives, are produced when amines react with di-tert-butyl decarbonate, or BOC. Consequently, during the synthesis of peptides, t-BOC serves as a protective group. The peptide's terminal amine is frequently protected by it. Amino acids can be added with boc groups by using a base and di-tert-butyl decarbonate. (Fig. 5).
Robust acids such as hydrochloride acid in methanol or trifluoroacetic acid (TFA) in dichloromethane can be used to eliminate it (William et al., 2003). N-methyl morpholine, triethylamine (TEA), or N-diisopropylethylamine (DIEA) may be utilized to neutralize the acids[61]. Both methods of peptide synthesis, SPPS and SPS, both employ boc protection groups.

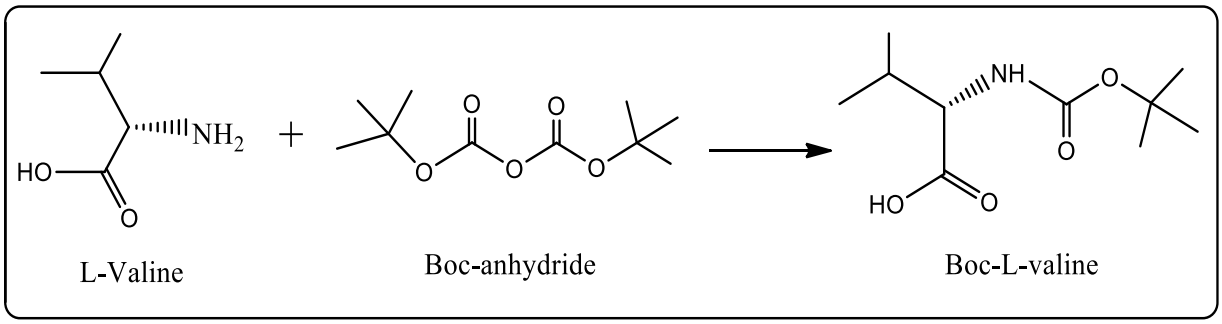


Figure 2.5: Boc-peptide coupling reaction (www.peptideguide.com)

2.6.2 Fmoc Protecting Group

Both SPPS and SPS prefer to use FMOC (fluorenylmethyloxycarbonyl) chemistry to protect the alpha amino group (Fig 2.5). Piperidine can cleave this group in extremely mild conditions, and it remains stable in acidic conditions.
Protected amino acid or peptide is first reacted with a base, such as piperidine, in solid phase synthesis in order to deprotect the FMOC and couple to the subsequent amino acid. N-terminus protecting group can be eliminated, and non-coupling reagents can be cleaned away. This permits the addition, in a similar manner, of subsequent desired amino acids or peptides to the existing peptide.

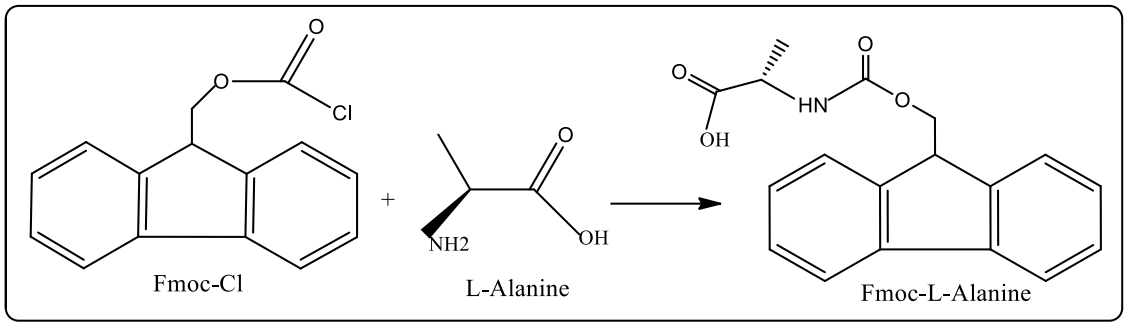


Figure 2.6: FMOC- peptide Coupling reaction (www.peptideguide.com)

2.7 APPLICATIONS OF FF-BASED NANOSTRUCTURES

Different functional nanostructures with potential applications in both biological and non-biological sectors were produced through the self-assembly of Phe-Phe based peptides. Tissue cell culture, medication administration, biosensors, bioimaging, and other technologies are included in the biological applications.

2.7.1 Biological Applications

Bio imaging-

Utilizing cationic dipeptides in conjunction with QDs to form 3D colloidal spheres is one example of how functional inorganic nanoobjects can be integrated into peptide systems to enable bioimaging. Their highly biocompatible nature and ability to be steadily distributed in a serum-containing cell medium are the outcome of a routine cytotoxicity test called MTT (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) cell-survival assay.

Biosensor applications have been demonstrated for peptide nanotubes that self-assemble from FF building blocks[62][63]. By depositing FNTs on a graphite electrode's surface, a new electrochemical biosensing platform is created, increasing the electrode's sensitivity [63]. When thiol modified FNTs are added to a Au electrode, that becomes responsive for a variety of enzymes, including ethanol dehydrogenase (ADH) and glucose oxidase (GOx)[64]. Based on the enzyme-related electrocatalytic reaction, these electrodes demonstrated enhanced sensitivity and repeatability for the detection of glucose and ethanol.

The following advantages make FNTs a valuable biomaterial for for the synthesis of sensors and biosensors

- Possess a great degree of stability
- Prompt detection period
- A significant density of current
- transport of electrons that is not facilitated

Nanofabrication

For instance, Ag ions reduction inside the nanotubes followed by peptide scaffolds enzymatic degradation and then insertion of thiol-containing peptide linkers may produce metal-peptide-metal trilayer coaxial nanocables with particular electromagnetic properties. Alternatively, 20 nm Au nanoparticles may covered over the FNTs surface coated with Ag nanowires[64]. The Pt-nanoparticle is templated by a nanotube made of the D-Phe-D-Phe dipeptide [40].

Drug Delivery –

Following the spontaneous conversion of self-assembled CDPNTs into vesicles, oligonucleotides could transported to the inside of cells by endocytosis, which alters the concentration of building blocks. Biocompatible, bioabsorbable, and recyclable are the delivery vehicles based on peptides. Thus, FF-based nanomaterials can be used for the delivery of foreign by creating new class of vectors.

Tissue engineering -

The extracellular matrix (ECM) for the three-dimensional (3D) cell culture has been informally employed in the fibrous hydrogel networks. Model cells called chondrocytes were mixed with the appropriate Fmoc-FF gel to incorporate them into hydrogels. The characteristics of certain chondrocytes were discovered by investigations using two-photon fluorescence microscopy and environmental scanning electron microscopy (ESEM), which shows the development and proliferation of cells in the three-dimensional fibrous networks [37].

Chapter 3

Methodology

3. MATERIALS AND METHODS

3.1 MATERIAL USED:

3.1.1 CHEMICALS USED:

Table 3.1: - List of Chemicals Used

Chemicals	Supplier
β-Alanyl-L-Histidine	SIGMA, ALDRICH, CO., USA
Chloroform	EMLURA®
Dichloromethane (DCM)	MERCK
Dimethylformamide (DMF)	MERCK
Trifluoracetic acid (TFA)	SIGMA, ALDRICH, CO., USA
N,N-Diisopropylethylamine (DIPEA)	SIGMA, ALDRICH, CO., USA
Benzaldehyde	LOBA CHEMIE PVT. LTD., INDIA
Glycine	REESOURCE CHEMICAL LTD.
Sodium acetate	S D. FINE CHEM. LTD., INDIA
Ethyl-acetate	MERCK
N-hydroxy Succinimide (OSu)	ALDRICH CHEM COMPANY
Tetrahydro-furan (THF)	SIGMA, ALDRICH, CO., USA
Di-cyclohexyl carbodiimide (DCC)	ALDRICH CHEM COMPANY
Methanol	EMLURA®
Ninhydrin	SIGMA, ALDRICH, CO., USA
Water	ELIX, MILI-Q
Hydrochloric Acid (HCl)	S D. FINE CHEM. LTD., INDIA
Meta- kresol	MERCK
Acetic anhydride	SIGMA ALDRICH, CO., USA
Fmoc-Protected amino acid	SIGMA, ALDRICH, CO., USA
Piperidine	SPECTROCHEM PVT. LTD., INDIA
Rink Amide and CTC- Resin	NOVABIOCHEM®
Diethyl-ether	QUALIGENS

3.2 TECHNIQUES USED

3.2.1 DYNAMIC LIGHT SCATTERING

Dynamic light scattering(DLS) which is also known as photo correlation spectroscopy. This technique is used for determination of particle size and charge of small particles in suspension and polymers in solution.

Principle of DLS: - This technique is based on the principle of Reyleigh scattering. Fine particles and molecules that are in constant random thermal motion called Brownian motion, diffuse at a speed related to their size. Particles that are small in size diffuse faster in comparison to particles that are larger in size.

The Zetasizer Nano-ZS is a high performance two angle particle and molecular size analyzer and analyze at very low or high concentration using dynamic light scattering with Non-Invasive Back Scatter technology (NIBS) optics. Zeta- potential indicates the degree of repulsion between adjacent similarly charged particles in dispersion. The ZS also incorporates zeta potential analyzer that uses electrophoretic light scattering for particles, molecules and surfaces and a molecular weight analyzer using static light scattering. Measurement of particle size on Zetasizer Nano-ZS (Malvern Instruments, Worcestershire, US) enlisting a nominal 4mW He-Ne laser operating at 633 nm wavelength.

Figure 3.1: Zeta Sizer

3.2.2 LYOPHILIZER

A lyophilizer, also referred as a freeze drier, is an instrument used in the food and pharma industries, among others, to remove water from products without disrupting their structural integrity. According to its fundamental concept, the material freezes and then put under vacuum pressure, which causes the water that has frozen to sublimate directly from the solid to the gas phase and leave behind a dry product. This technique avoids the damage that conventional drying techniques impose on fragile material. Lyophilizers are used to store and transport perishable commodities more easily, preserve product integrity, and increase the shelf life of heat-sensitive materials. The steps included in this fundamental process are detailed as follows:

a) Freezing: The product is frozen. It is an essential requirement for low-temperature drying operation. Applications can continuously be at temperatures close to absolute zero.

b) Vacuum: The product is placed under several degrees of vacuum. This allowed the frozen sample in the product is to 'vaporize' without first passing through the liquefaction stage: a process named sublimation. It is the requisite heat in dissipation source to evaporate the ice in a feebly heated space heaters.

c) Condensation: Low-temperature condenser plates. The vaporized solvent is conveyed through a heat exchanger powered by a refrigeration or refrigerating principle. It converted back to a solid and removed from the vacuum chamber. The separation operation is now accomplished.

Figure 3.2: Lyophilizer

3.2.3 CENTRIFUGE

A centrifuge is an instruments used in labs to separate liquid mixture components according to their density. Its working principle relies on employing centrifugal force, which is produced

whenever the sample rotates quickly. Denser particles remain toward the tube's bottom while the centrifuge spins, while lighter particles remain suspended or rise toward the top. It can be achieved to distinguish specific components from complex mixtures, such as cells, proteins, or nucleic acids, due to this technique.

Many scientific disciplines, including biochemistry, microbiology, and medical diagnosis, utilize centrifuges significantly. Centrifugation is a technique used by researchers to separate different phases of chemical or biological reactions, concentrate analytes, and purify products. Centrifuges serve a purpose in medical contexts for processing blood samples, extracting serum or plasma from cellular components, and conducting diagnostic test. Reaction mixture was filled in centrifuge tubes , spun at 8000 rpm for 10 min at 4°C. Heraeus® Multifuge® 3SR plus Centrifuge was used to sperate out peptides from oil and impurities.

Figure 3.3: Centrifuge

3.2.4 UV-VISIBLE SPECTROSCOPY

The working of UV-visible spectroscopy based on the principle of Beer-Lambert law, which states that "*when a beam of monochromatic light is passed through a solution of an absorbing substance, the rate of decrease of intensity of radiation with thickness of the absorbing solution is proportional to the incident radiation as well as the concentration of the solution*"[65]. The technique of UV-visible spectroscopy is used for studying how matter reacts with both ultraviolet and visible light. It measures the amount of light a sample absorbs, transmits, and reflects throughout a range of UV and visible spectrum wavelengths. Several scientific fields, including chemistry, biochemistry, ecological science, and materials research, use UV-visible spectroscopy. All the UV- spectroscopy measurements were carried out on Cary-60 (USA).

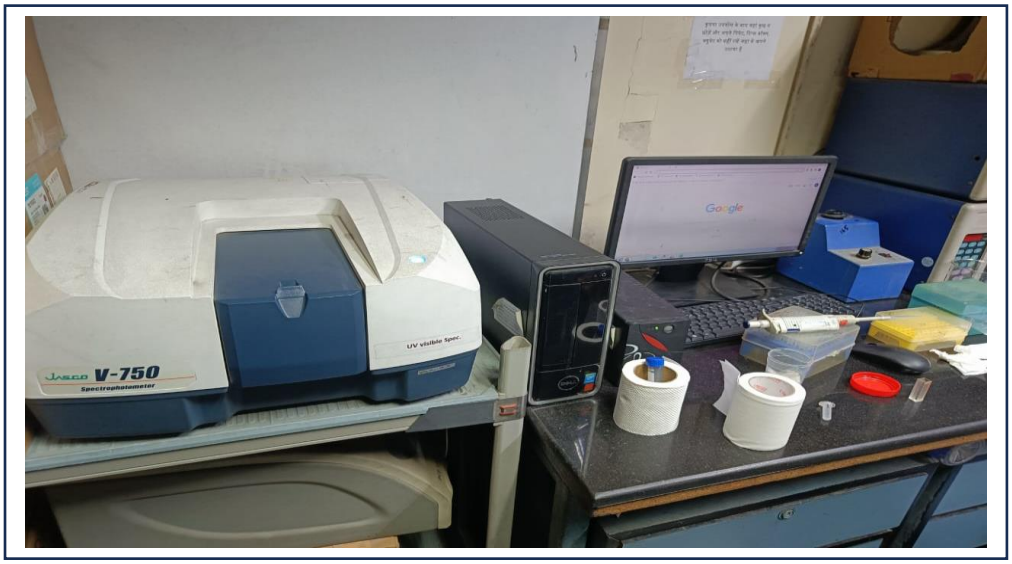


Figure 7: UV-Visible Spectrophotometer

3.2.5 THIN LAYER CHROMATOGRAPHY

TLC is a commonly used chromatographic method which utilizes a thin layer of an adsorbent substance to facilitate the migration of molecules at varying rates for the purpose of separation and analysis. In TLC, just a small amount of the compound mixture is applied on a stationary surface. This involves typically a thin layer of alumina or silica gel which has been placed on a glass or plastic plate. The plate is then placed in a chamber which contains a solvent that rises to the plate as a result of capillary action. In the process, chemical elements are carried along by the solvent and separate based on their polarity due to their unique interactions with the solvent and stationary phase. TLC is used as a preliminary technique for chemical isolation, which makes later analysis easier.

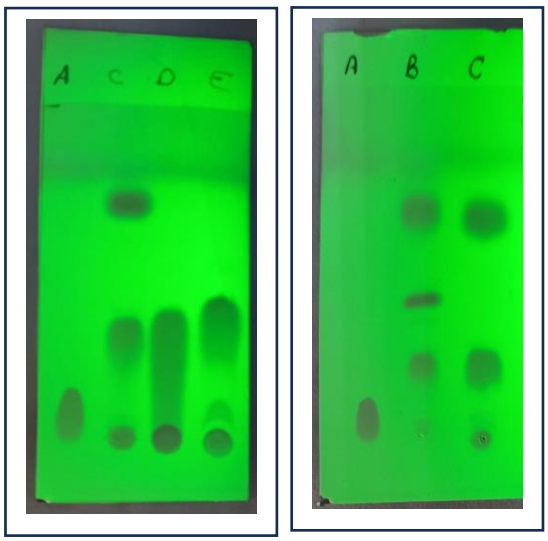


Figure 3.5: Thin layer chromatography

3.2.6 FT-IR SPECTROSCOPY

An effective analytical technique for identifying and characterizing chemical compounds based on their responses to infrared light is Fourier Transform Infrared (FT-IR) spectroscopy. It works on the basis of the idea that molecules absorb infrared light at specific frequencies which correspond to the modes of vibration of their chemical interactions. A device that controls the infrared radiation intensity over time during FT-IR spectroscopy, producing an interferogram. When this interferogram is Fourier transformed, a spectrum representing the sample's absorption of infrared light appears. It is essential for qualitative and quantitative analyses of organic and inorganic compounds because it makes it easier to identify functional groups and understand molecular structures. Single beam Perkin Elmer spectrophotometer (Spectrum BX Series), USA was used to carried out parameters.

Figure 3.6: FT-IR Spectrophotometer (usic.du.ac.in)

3.2.7 NMR SPECTROSCOPY

NMR spectroscopy is a powerful analytical technique which utilizes the magnetic properties of atomic nuclei to show the complex details of molecular behavior and structure. It works on the concept that atomic nuclei with an odd number of protons or neutrons have a magnetic moment, which aligns with an external magnetic field. When nuclei are exposed to radiofrequency radiation in the presence of a magnetic field, they resonate and change states of energy. By analyzing the frequencies and intensities of these resonance signals, useful information into the molecular chemical environment, connection, and conformation can be gained. It allows for the exact identification of the structures of organic and inorganic compounds, as well as the unraveling of molecular relationships and the investigation of reaction mechanisms. ^{1}H-NMR spectra of the compounds were collected on Bruker 400 MHz spectrophotometer.

Figure 8: NMR (www.chem.pitt.edu)

3.2.8 MASS SPECTROSCOPY

Mass spectrometry is an important spectrometry technique used to determine mass-to-charge ratio of ions, providing crucial insights into the molecular structure, content, and the majority of compounds. MS involves ionizing a sample to produce charged particles. It is based on the idea that ions may be separated according to their mass-to-charge ratio when subjected to electric and magnetic fields. Within the fields of the mass spectrometer, these particles accelerates and deflect. The ions generated are then recognized and measured, producing a mass spectrum demonstrating the distribution of ions according to mass-to-charge ratio. Many scientific fields, including biology, chemistry, and environmental research, use this technique extensively. It simplifies the process of characterizing biomolecules including proteins and peptides, quantifying analytes in complex mixtures, and identifying unknown substances. LC-MS with triplquadrupole Quattro micro API, Waters Inc. USA was used to carried out mass spectra of compounds.

Figure 3.8: Mass Spectroscopy (mcac.umbc.edu)

3.2.9 HIGH PERFORMACE LIQUID CHROMATOGRAPHY (HPLC)

HPLC is widely utilized in analytical chemistry to separate, identify, and quantify components in mixtures. Based on chromatographic principles, HPLC runs the sample combination through a column that has a stationary phase after it was successfully dissolved in a solvent. Based on different chemical properties, the stationary and mobile phases' interactions with the sample components lead to better separation.

HPLC has greater advantages than traditional liquid chromatography because it can withstand high pressures, resulting in separations faster and more effective. The food and beverage industry, pharmaceuticals, environmental monitoring, forensic research, and other fields all rely heavily on HPLC, which is highly recognized for its exceptional sensitivity, resolution, and versatility. Its accuracy in evaluating intricate mixes and identifying minute substances is what accounts for its adaptability.

Figure 9: HPLC (media.labcompare.com)

3.2.10. TRANSMISSION ELECTRON MIROSCOPY (TEM)

TEM is a powerful imaging method that examines at the internal structure and composition of materials at the nanoscale level by using a concentrated beam of high-energy electrons. By passing an extremely thin specimen through the electron beam, this potent microscopy technique produces an image of the sample based on the variations in electron transmission and scattering. The fundamental principle of TEM involving the ability of electrons to pass through thin material because of their short wavelength. The specimen's atoms are impacted by the electrons as they go through it, revealing intricate details about the sample's shape, chemical makeup, and internal structure. TEM is widely used in many sectors, including materials science, nanotechnology, biology, and medicine, since it allows researchers to

analyze the fine details of structures, flaws, and chemical composition on the nanoscale scale. Techni G2 30 U-twin, Technai 300 kV ultra-twin microscope is used in this research work.

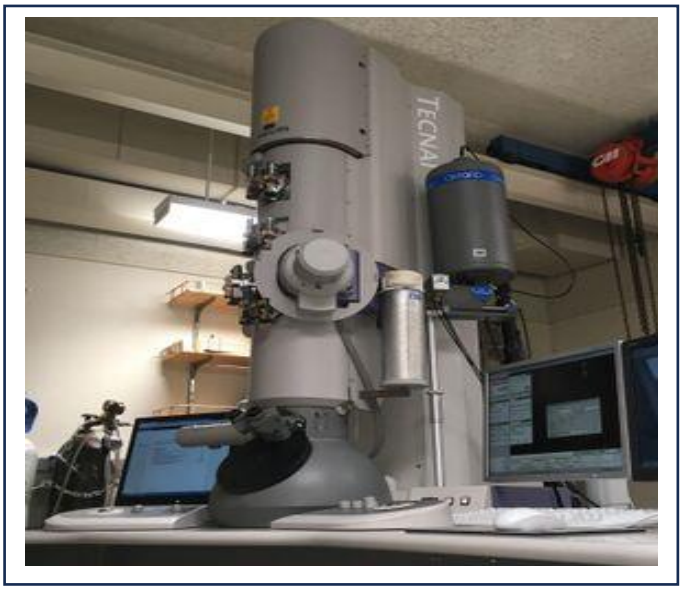


Figure 3.10: TEM (lab.kni.caltech.edu)

3.3 METHOD USED

3.3.1 SOLUTION PHASE PEPTIDE SYNTHESIS

Ac-ΔPhe-Pro-βAla-His-NH_2(I)

Ac-ΔPhe azlactone:-

In a solution of N-acetylglycine (9.15 g, 86 mmol), sodium acetate (6.45 g, 78.6 mmol), and benzaldehyde (7.25 g) in ethyl acetate (18.75 mL), acetic anhydride (5.4 g) was added. The mixture was heated for 20 hours at a temperature ranging from 85°C to 90°C with constant stirring. The reaction mixture was kept at 5°C for cooling. Subsequently, deionized water(382ml) was added to precipitation of desired product and allowed to cool at 5°C for an hour. The final product was filtered and characterized by UV spectroscopy (UV: 280 nm).[66]

Ac-ΔPhe-Pro-OH :-

To a solution of Ac-ΔPhe azlactone (2.25 g, 11.9 mmol) in THF (20 ml), a solution of sodium salt of proline (2.5 gm, 18.2 mmol) in 10 ml of water was added at 5°C with stirring. After completion of reaction, the THF was removed at reduce pressure and reaction mixture was further acidified using HCl. The product was recovered by vacuum filtration and dried.

Ac-ΔPhe-Pro-βAla-His-OH (I) :-

To a solution of Ac-ΔPhe-Pro-OH (302mg,1mmol) and OSu (138.108 mg,1.2 mmol) in tetrahydrofuran (20 ml THF), DCC (1 mmol, 206 mg) was added at chilled conditions (ice bath). The reaction mixture was stirred on ice for 30 minutes and a solution of β-alanyl-L-histidine (226mg, 1 mmol,) and (1 mmol, 53mg) Na_2CO_3(53mg, 1 mmol) in 20 ml water and stirring for overnight. TLC was performed to confirm the reaction completion. After completion of reaction the DCU the white byproduct (DCC) was filter out and THF solvent was removed on vaccuo. Remaining aqueous solution was washed with ethyl acetate (3X20ml). Acidify the solution using HCl and remove the water solvent under vacuuo. Adding methanol allowed the salt to settle. Centrifuge the solution, methanol was removed leaving behind a crystal-like structure (the compound with OSu). To remove the OSu, add 2 ml of ethyl acetate, as OSu is soluble in it. Repeat this step. Dissolve the remaining solid compound in methanol and perform TLC to check the separation. Centrifuge the methanol solution and remove the methanol on vaccuo Dry the final peptide product, Ac-ΔPhe-Pro-β-Ala-His-OH, 813.5 mg (68% yield). UV: 220nm, 280 nm. Product was purified by HPLC.

3.3.2 SOLID PHASE PEPTIDE SYNTHESIS

Ac-ΔPhe-Pro-Arg-βAla-His-OH(II) and **Ac-ΔPhe-Pro-Arg-βAla-His-NH_2(III)** were synthesized by solid-phase peptide synthesis (SPPS) method using Fmoc- chemistry[67]. Peptide carboxylic free peptide (**II**) was synthesized using 2-chlorotrityl chloride, while rink amide resin was utilized for making amide peptide (**III**).

Ac-ΔPhe-Pro-Arg-βAla-His-OH(II)

To synthesize **II** peptide, weigh 500 mg of 2-chlorotrityl chloride resin and swell in dichloromethane (DCM) for 4 hours. Protected amino acid Fmoc-His(Trt)-OH (310mg, 0.5 mmol) was loaded onto the resin by anhydrous DMF (3.0 ml) containing DIPEA (209 µl, 2.4 mmol) for overnight. Initially, the capping solution (CH_2Cl_2 : MeOH: DIPEA, 17:2:1, v//v/v) was used to cap the unreacted triacyl chloride moieties on the resin[68]. Three 10-minute each treatment with 20% piperidine in DMF (3X3.0 ml) was given to deprotect Fmoc group. Ninhydrin test was performed to confirm the deprotection. The next amino acid, Fmoc-βAla-OH (561 mg, 0.5 mmol), was linked to the free amino group using the coupling reagent, COMU (214 mg, 0.5 mmol), and DIPEA (522 µl, 2.4 mmol) for three hours. The above steps were repeated until the final amino acid, Ac-ΔPhe-OH, was attached. Following each stage of deprotection during peptide synthesis, the ninhydrin assay was carried out. The final compound **II** peptide was obtained by cleaved and deprotection of side chain of Arg and His by 95% TFA in m-cresol (3.0 ml) for three hours. After adding 50 ml of diethyl ether to the mixture, the precipitate was collected, cleaned with 3 x 50 ml of ether, and vacuum dried.

Ac-ΔPhe-Pro-Arg-βAla-His-NH_2(III)

Rink amide resin (~500 mg) was swelled in DCM for the production of **III** peptides, and the Fmoc group was then deprotected using 20% piperidine (as previously mentioned). Subsequently, the first amino acid, Fmoc-His(Trt)-OH (309.85 mg, 0.5mmol), was loaded using a coupling agent, COMU (155.5 mg, 0.5 mmol), and DIPEA (260.5 µl, 3mmol) as base. Capping solution for resin was prepared adding acetic anhydride in DMF (~2.0 ml, 2:16, v/v). The resin was capped preparing solution of acetic anhydride in DMF (~2.0 ml, 2:16, v/v). The same method was used for stepwise deprotection and coupling of the amino acids, using 2.0 meq of the starting amino acid, base, and COMU until the required sequence was synthesized[68]. TFA (85%) with DCM(10%) and H_2O(5%) in 2.0 ml of m-cresol was used to cleave the M-NH2 peptides from the resin, and the process was built up as mentioned above.

Yield of Ac-ΔPhe-Pro-Arg-βAla-His-OH and Ac-ΔPhe-Pro-Arg-βAla-His-NH_2 is 30 % and 60% in the form of white powder. Synthesized peptides are characterized using various characterization techniques.

3.3.3 NANOSTRUCTURES FORMATION

To induce self-assembly of peptide for generating nanostructures, disperse peptide in 100µl methanol followed by 900µl water with vigorous vertexing for 2-3 minutes. Leave for 2-3 hours to self-assemble.

3.3.4 DYNAMIC LIGHT SCATTERNG (DLS) STUDIES

Characterization of hydrodynamic size and surface charge of synthesized dehydro-peptides Ac-ΔPhe-Pro-βAla-His-OH (**I**), Ac-ΔPhe-Pro-Arg-βAla-His-OH (**II**) and Ac-ΔPhe-Pro-Arg-βAla-His-NH_2 (**III**), was carried out by DLS. The hydrodynamic diameter was measured in triplicates and average was taken at angle of 173° forward scatter.

3.3.5 UV-VIS SPECTROSCOPY

0.1 mg/ml methanolic solution was prepared for absorbance spectrum studies. It was measured at the wavelength range of 200-300 nm.

3.3.6 TRANSMISSION ELECTRON MICROSCOPY (TEM)

With the Technai 300 kV ultra-twin microscope, images were captured utilizing the Technai G2 30 U-twin. Samples were directly spotted onto copper grids with 200 mesh carbon coating, and were left to stand for one minute. After carefully removing excess solvent, the grids were immediately negatively stained with Uranyl acetate (20µL). For 15 to 20 minutes, the grids were air dried.

3.3.7 NMR SPECTROSCOPY

10mg/ml solution of deuterium methanol (d-methanol) was used for the characterization study.

3.2.8 ZONE OF INHIBITION (ZOI) ASSAY

As mentioned in the experimental section, the Kirby-Bauer disk susceptibility test was employed to evaluate the antimicrobial activity of all three of the synthesized dehydro-peptides. Gram-positive strains, *Staphylococcus. aureus*, and gram-negative strains, *Klebseilla pneumoniae*, were used as test organisms. Mueller Hinton agar plates were coated with bacterial culture (OD600: 0.4-0.8). Susceptibility disks with a diameter of 6.0 mm were added to the cultured agar plates and then soaked in peptide solutions (250 µg per disc). The plates were then incubated for 24 hours at 37°C. The positive and negative controls were streptomycin (20 µg per disk) and autoclaved water, respectively. Using a Vernier Caliper, around each filter paper disc area of inhibition was measured. Three duplicates of each experiment were performed to ensure accuracy.

3.2.9 MINIMAL INHIBITION CONCENTRATION (MIC) ASSAY

Resistance microorganisms, *S. aureus and K. pneumoniae*., were used to determine the minimal inhibitory doses of produced dehydro-peptides. In summary, 200 µl of MHB media were inoculated with 10 µl of SA and KP bacterial cultures with an OD600 of 0.4 on 96-well plates.

Following that, varying quantities of peptide samples (300–1200 μg/ml) were added. Following a 12-hour incubation period at 37°C, 30 microliters of p-iodonitrotetrazolium violet (INT) dye were added to each well, and the plates were left to incubate for an additional 30 minutes at 37°C. The development of color was noted as a micro-organism development sign. Untreated bacterial cells were regarded being the negative control. MIC values were calculated using the lowest concentration with no color modification after 30 minutes of INT dye addition.

Chapter 4

Result & Discussion

4.1 Synthesis of Ac-ΔPhe-Pro-βAla-His-OH (I) , Ac-ΔPhe-Pro-Arg-βAla-His-NH_2(II) & Ac-ΔPhe-Pro-Arg-βAla-His-OH (III)

Ac-ΔPhe-Pro-βAla-His-OH (**I**) was synthesized by standard solution phase peptide synthesis and Ac-ΔPhe-Pro-Arg-βAla-His- OH (**II**) and Ac-ΔPhe-Pro-Arg-βAla-His- NH_2 (**III**) were synthesized by solid phase peptide synthesis reported in methodology section using Fmoc chemistry. After purification by HPLC, the purified peptide was characterized by various spectroscopy techniques.

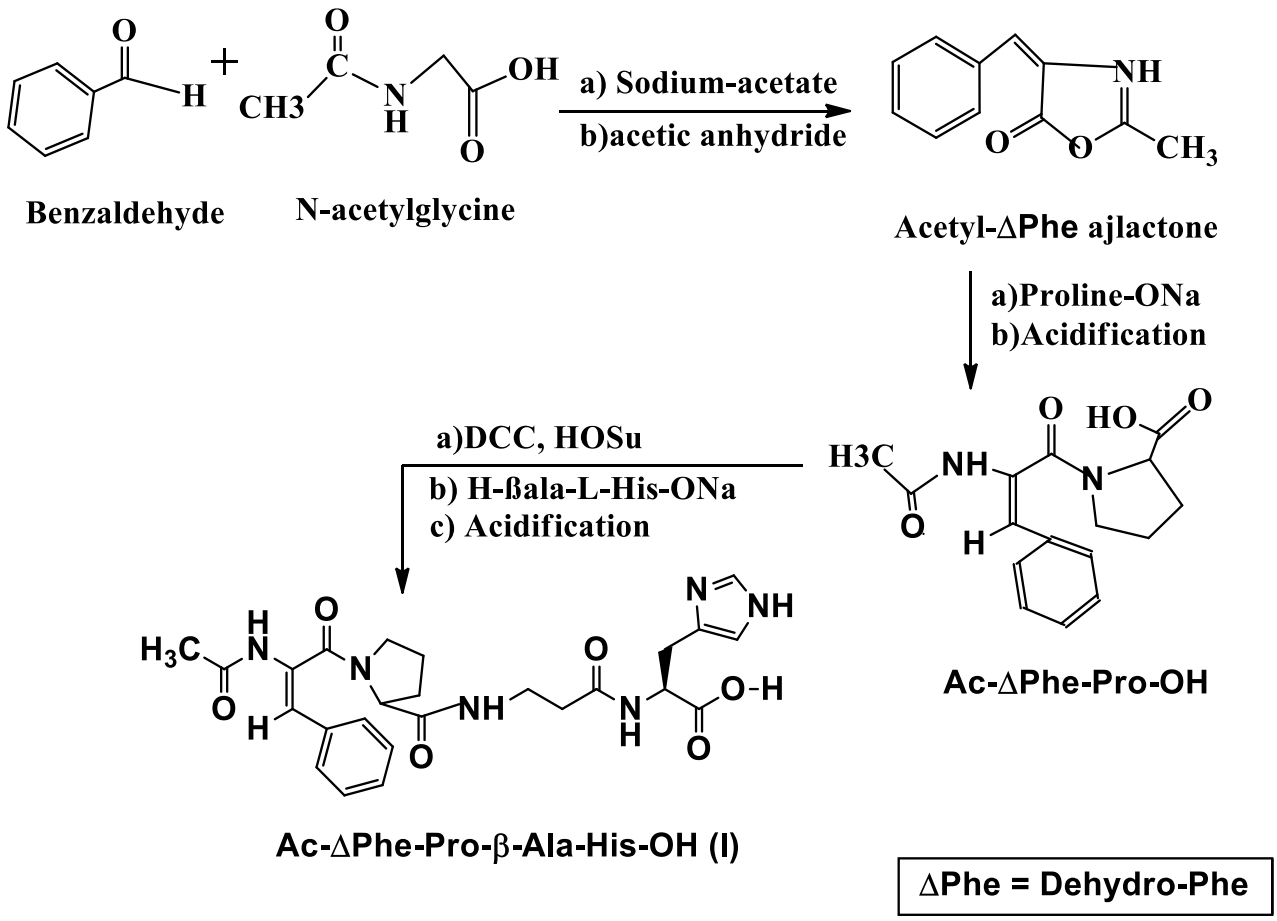


Figure 4.1: Schematic representation of solution phase synthesis of dehydro-peptide

Synthesis of Ac-ΔPhe-Pro-Arg-βAla-His-NH_2 (II) and Ac-ΔPhe-Pro-Arg-βAla-His-OH (III) :

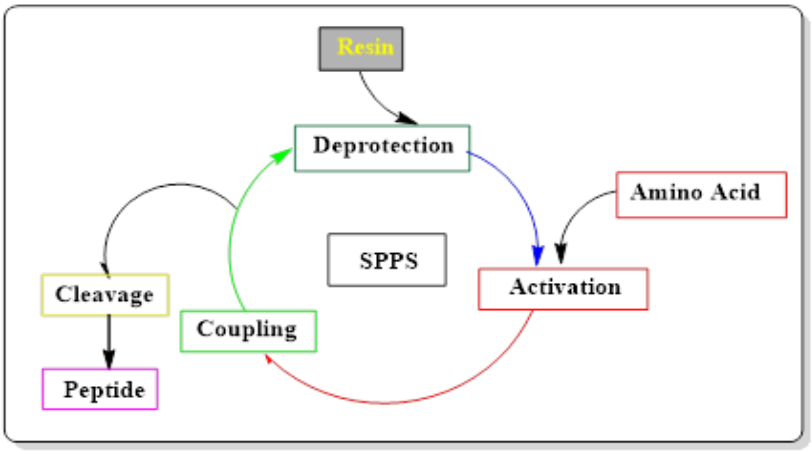


Figure 4.2: Schematic representation of solid phase synthesis of dehydro-peptide

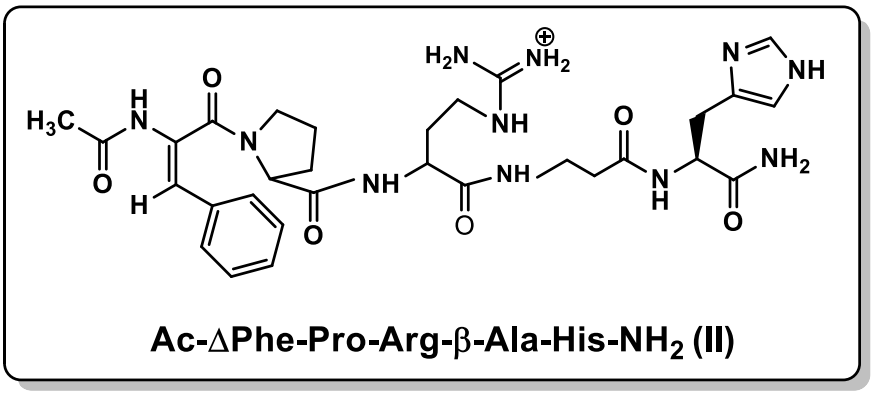


Figure 4.3: Chemical Structure of Ac-ΔPhe-Pro-Arg-βAla-His-NH_2 (II)

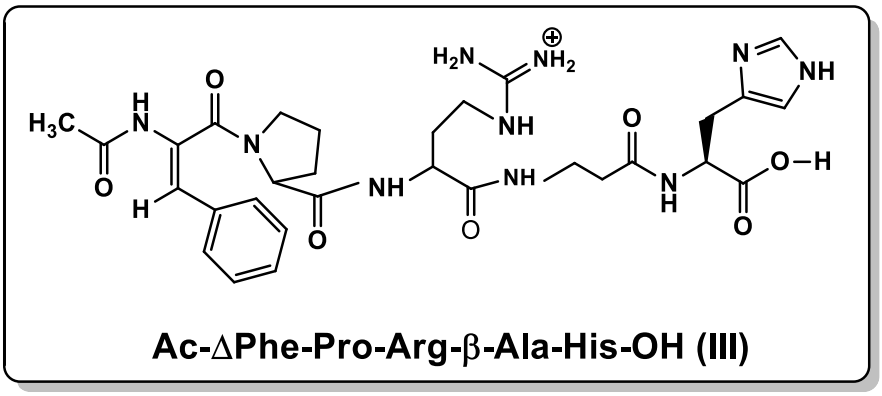


Figure 4.4: Chemical Structure of Ac-ΔPhe-Pro-Arg-βAla-His-OH (III)

4.1.1 PURIFICATION BY HPLC

HPLC purification of synthesized peptides (**I**, **II**, **III**) was carried on RP C-18 coloumn using water- acetonitrile gradient containing 0.01% TFA. HPLC data of Ac-ΔPhe-Pro-βAla-His-OH (I), Ac-ΔPhe-Pro-Arg-βAla-His-OH (II), Ac-ΔPhe-Pro-Arg-βAla-His-NH_2 (III). Peak of peptides at RT ~12 min are showing in following figures.

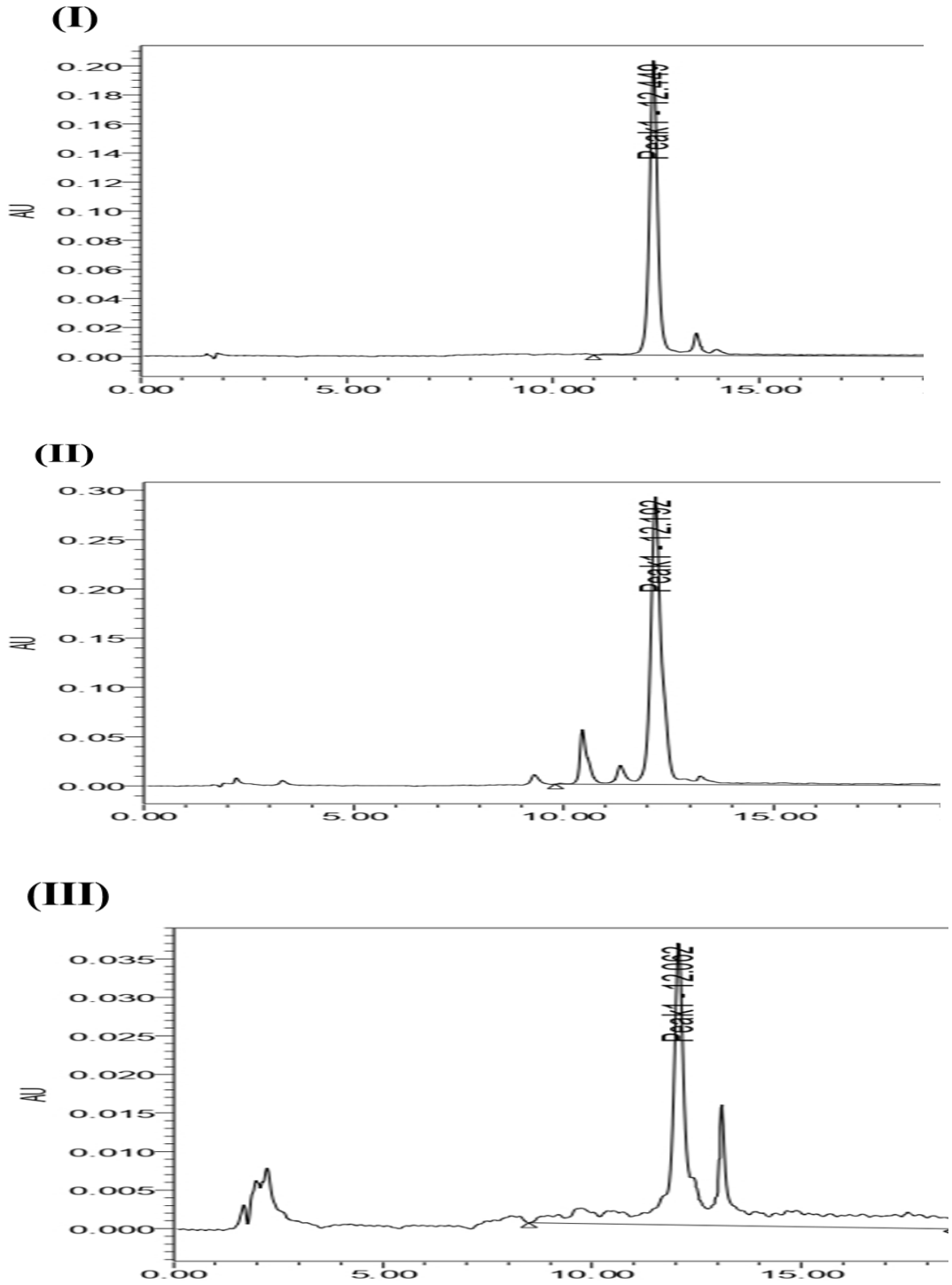


Figure 4.5: HPLC Profile of Nanostructures

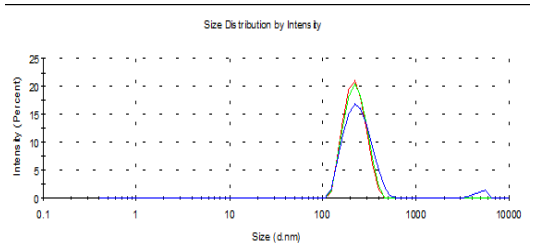
4.1.2.1 DYNAMIC LIGHT SCATTERING STUDIES

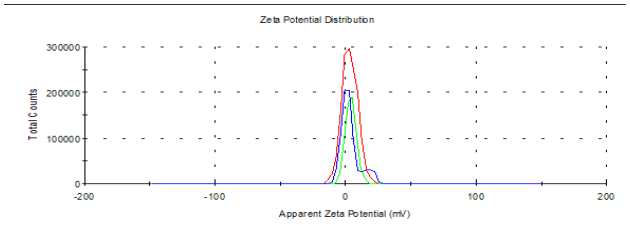
In dynamic light scattering studies, synthesized peptide were self-assembled in aqueous media in the range of 222nm-600nm with a positive zeta potential ranging from (+4) to (+9) Table (4.1).

Table 4.1: Particle Size of Nanostructures

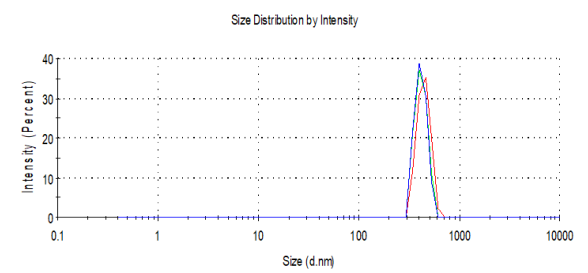
Sample	Avg. particle size (d.nm) ± S.D.	Zeta potential (mV) ± S.D.
Ac-ΔPhe-Pro-βAla-His-OH (I)	222.7 ± 15.73	4.56 ± 1.92
Ac-ΔPhe-Pro-Arg-βAla-His-OH (II)	749.9 ± 93.08	9.06 ± 1.66
Ac-ΔPhe-Pro-Arg-βAla-His-NH_2 (III)	559.8 ± 73.65	8.16 ± 1.92

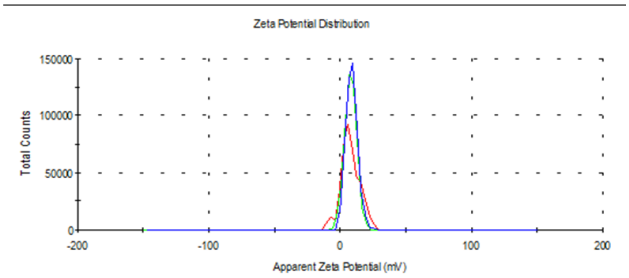
(I)



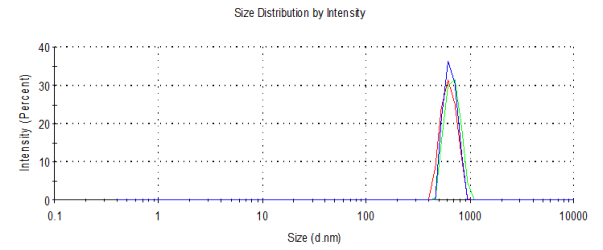


(II)





(III)



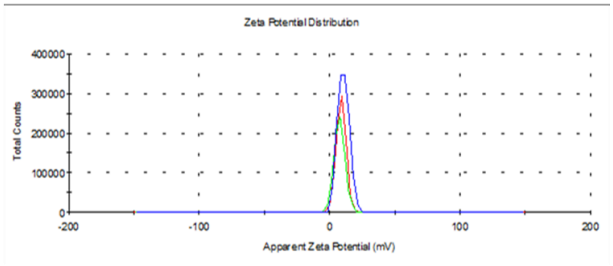


Figure 4.6: Size and Zeta potential of deydro-peptide based nanostructures.

4.1.2.2 UV-DATA OF NANOSTRUCTURES

UV- Spectrum of nano-structures I, II & III showed the peak of peptide bond and dehydro-Phe which confirms that the synthesis was successfully completed.

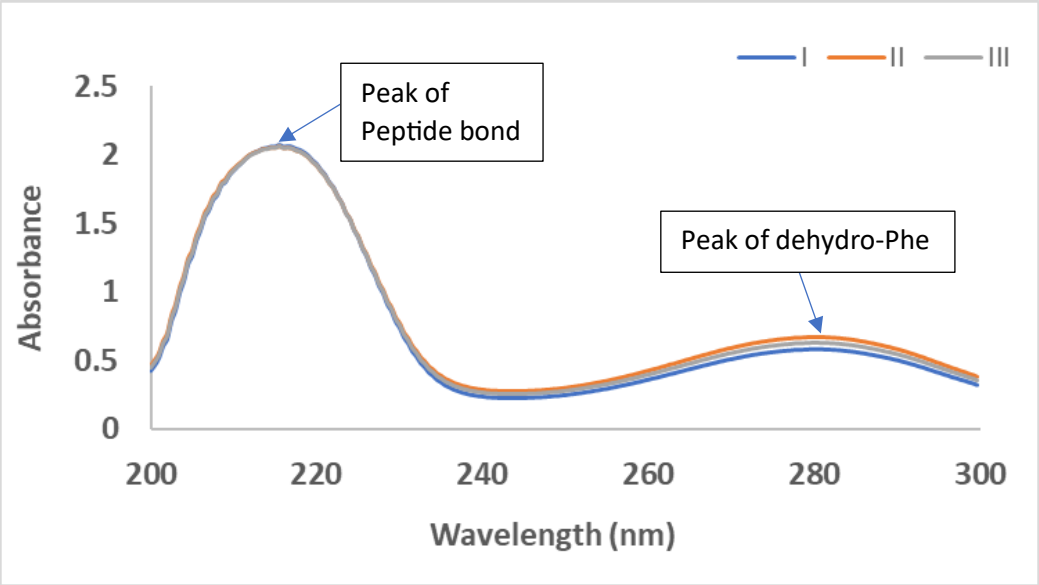


Figure 4.7: UV-data of dehydro-peptide based Nanostructures.

4.1.2.3 NMR DATA OF NANOSTRUCTURE

NMR data of Ac-ΔPhe-Pro-βAla-His-OH (I) is shown below. Graph showing peak of aromatic hydrogen which confirms the synthesis. NMR data of remaining nanostructure is under processing.

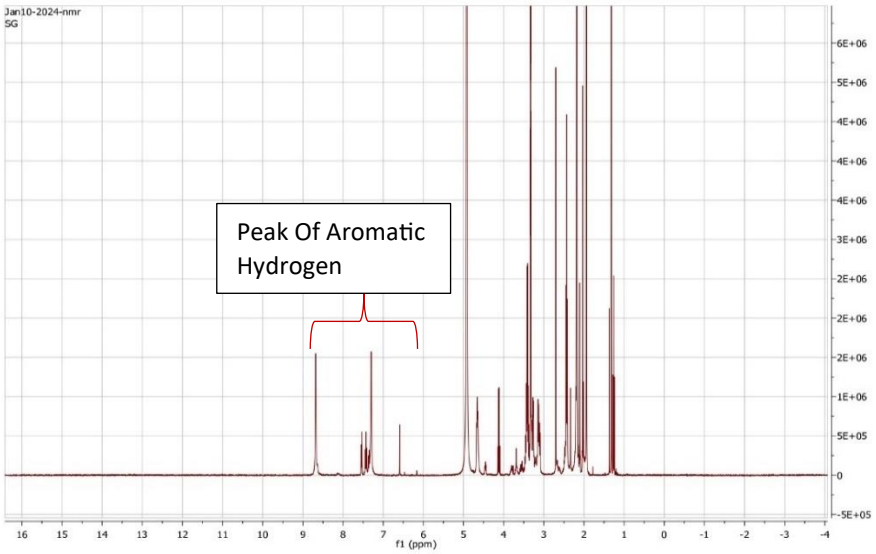


Figure 4.8: NMR Spectra of Ac-ΔPhe-Pro-βAla-His-OH (I)

4.1.2.4 FT-IR DATA OF NANOSTRUCTURE

IR data of Ac-ΔPhe-Pro-βAla-His-OH (I) is shown below. Graph Showing the peak around ~3400 cm-1 confirms the presence of N-H bond, peak around ~1700-1750 cm-1 confirms the presence of carboxylic group. FT-IR data of remaining nanostructure is under processing.

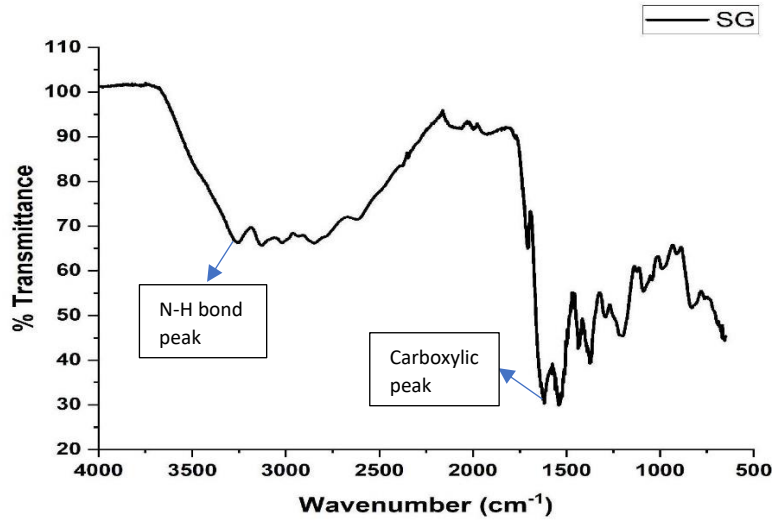


Figure 4.9: FT-IR Spectra of Ac-ΔPhe-Pro-βAla-His-OH (I)

4.1.3.5 MASS SPECTRUM OF NANOSTRUCTURE

Mass spectra of Ac-ΔPhe-Pro-βAla-His-OH(I) shown below. Mass peak at 511.23 confirm the peptide **I**. Mass data of remaining nanostructure is under processing.

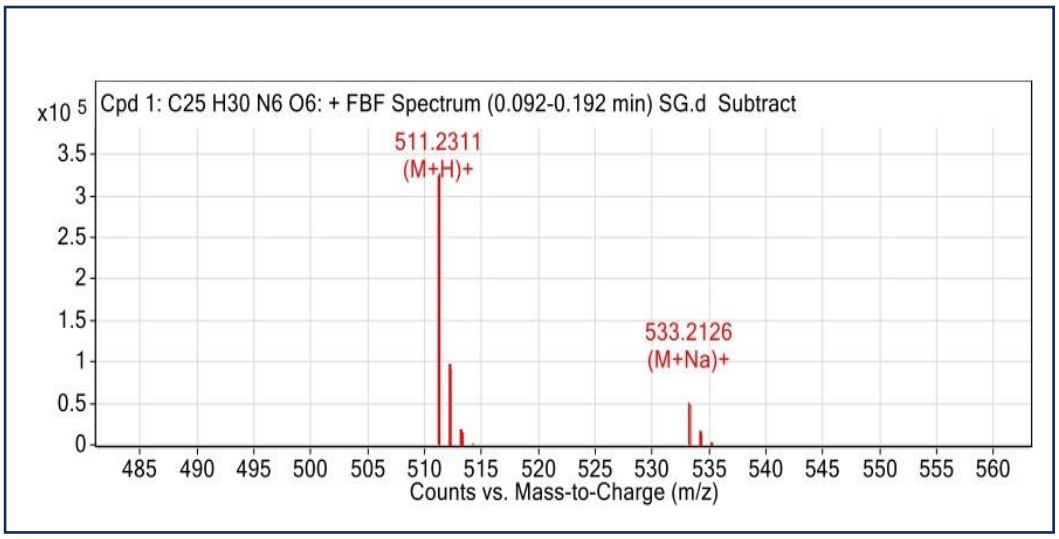


Figure 4.10: Mass spectrum of Ac-ΔPhe-Pro-βAla-His-OH (I)

4.1.2.6 TEM-IMAGE OF NANOSTRCTURE

The shape and size of the peptide nanostructures were also studied using TEM. The self-assembled peptide nanostructures showed spherical shaped particles with ~98 nm in size. Smaller nanostructures were found in TEM than in DLS; variation in width could explained by the fact that hydrodynamic diameter of the particles measured by DLS, whilst TEM provides the size in the dried condition. TEM- Image of remaining dehydro-peptide based nanostructures is under processing.

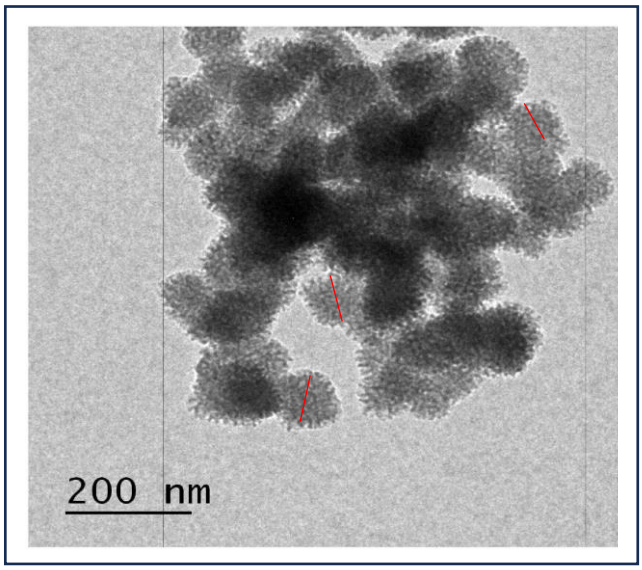


Figure 4.11: TEM-Image of Ac-ΔPhe-Pro-βAla-His-OH (I)

4.2 ANTIMICROBIAL ACTIVITY

4.2.1 MICROORGANISMS AND GROWTH CONDITIONS

Bacteria strains, S. aureus, and K. Pseudomonas were maintained at 37 ◦C in Luria Bertani (LB) media.

4.2.2 ZONE OF INHIBITION ASSAY

The antimicrobial activity of nanostructures was determined by using Kirby-Bauer single disc susceptibility test. Zone of Inhibition is a quick and qualitative method of determining an antimicrobial agent's capacity to suppress the development of microorganisms.

Among all the synthesized peptides the antimicrobial activity was shown by peptide (II) only.

(a) *S. Aureus* (b) *K. Pseudomonas*

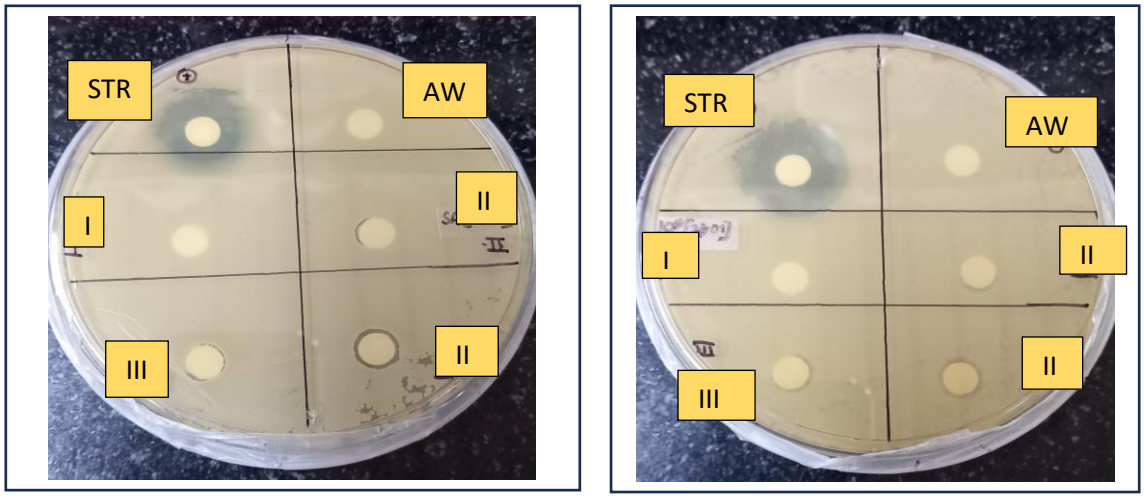


Figure 4.12: Zone of inhibition assay of dehydro-peptides (I, II, III) on gram-positive and gram-negative bacteria using autoclaved water as negative control and streptomycin as positive control.

4.2.3 DETERMINATION OF RELATIVE MINIMUM INHIBITORY CONCENTRATION (MIC)

From the result it was observed that peptide (II) showing initial results of relative inhibitory concentration.

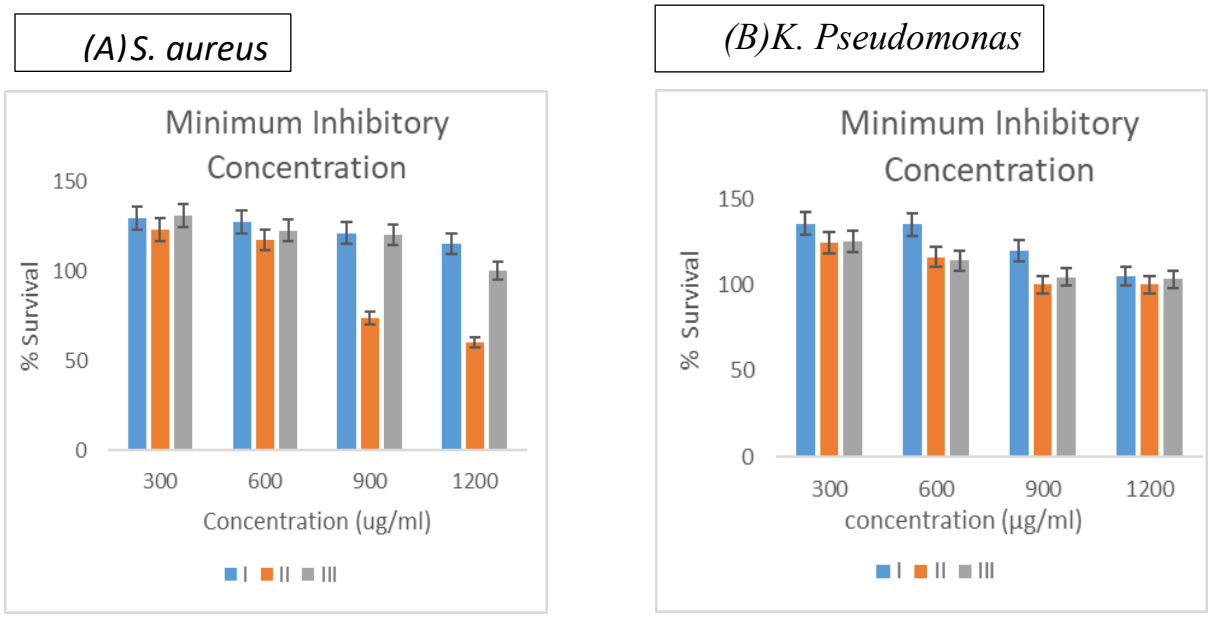


Figure 4.13: Relative Minimum Inhibitory Concentration of dehydro-peptides(I, II, III), on gram-positive and gram-negative bacteria using autoclave water as negative control and streptomycin as positive control.

Chapter5

Conclusion & Future Scope

We synthesized and bio-physical characterization new imidazole containing dehydro-peptide-based nanostructures, Ac-ΔPhe-Pro-βAla-His-NH2(**I**), Ac-ΔPhe-Pro-Arg-βAla-His-OH(**II**), and Ac-ΔPhe-Pro-Arg-βAla-His-NH2(**III**), that include the α, β-dehydro-phenylalanine residue. Introducing ΔPhe is crucial for increasing proteolytic stability via establishing structural restrictions on the peptide backbone. These peptides self-assembled in aqueous media to form nanostructures in range of 222nm-600nm with a positive zeta potential ranging from (+4) to (+9). Antimicrobial experiment studies, showed peptide II show initial antimicrobial activity.

Overall, this study establishes the foundation for future advances in self-assembled nanostructures, particularly those based on dehydro-amino acids, and brings up potential for application in novel therapeutic techniques. The effectiveness of this research shows the enormous potential of these created nanostructures in biotechnology and medicine.

Chapter 6

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