Peptide Mediated Co-assembly of Porphyrin: Towards Sustainable Biomaterials for Light Harvesting and Catalysis

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PEPTIDE MEDIATED CO-ASSEMBLY OF PORPHYRINS: TOWARDS SUSTAINABLE BIOMATERIALS FOR LIGHT HARVESTING AND CATALYSIS

by

WSM NADEESHA KANCHANA KUMARI WIJERATHNE

A dissertation submitted to the Graduate Faculty in Chemistry in partial fulfillment of the requirements for the degree of Doctor of Philosophy,

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Peptide Mediated Co-assembly of Porphyrin: Towards Sustainable Biomaterials for Light Harvesting and Catalysis

By

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This manuscript has been read and accepted for the Graduate Faculty in Chemistry in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

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THE CITY UNIVERSITY OF NEW YORK
DEDICATION

To My Mother, Father, Husband and my two lovely sons
The diverse molecular functions of naturally occurring biomaterials designed from proteins are fundamentally based on a set of conserved building blocks, namely the 20 gene coded amino acids. The supramolecular structures and functions of proteins dictates by the self-assembly, where the complexity of proteins arise from a large number of amino acids. Generation of biomimetic systems that resemble the structures and functions of proteins is of great interest yet challenging due to the tremendous complexity of the natural systems. It is important to investigate alternative strategies to design much simpler systems that exhibit the same or similar function as proteins. Short peptides and aromatic peptide amphiphiles (2-5 amino acids short) are ideal candidates to generate minimalistic functional versions of complicated biological systems with same function and biocompatibility.

Thermodynamically driven fully reversible self-assembly of peptides is one the three main strategies (in addition to kinetically controlled and out-of-equilibrium assemblies) employed to create peptide based supramolecular structures. The fully reversible self-assembly of aromatic peptide amphiphiles via enzymatic hydrolysis and condensation of precursors to form peptide bonds in-situ can generate thermodynamically optimized conformations. These well-ordered nanostructures can act as scaffolds to effectively incorporate functional molecules (porphyrins as explained in chapters 3-5), via non-covalent interactions to generate soft nanomaterials. The co-assembled nanostructures get stabilized by hydrogen bonding interactions between amide backbones, and further stabilized by π-stacking interactions of aromatic moieties, with porphyrin molecules co-assembled into the aromatic stack. The resulting supramolecular structures organize porphyrin molecules inside the peptide nanostructures in such a way that they can facilitate the
energy transfer by fulfilling the required spatial arrangement through highly ordered peptide nanostructures.

Cyclic dipeptides are simple yet versatile molecules with customizable supramolecular properties, which are dictated by the functionality of their two amino acid side chains. Their self-assembly propensity has typically been investigated using cyclic dipeptides obtained by separate chemical synthesis or other pathways not always compatible with self-assembly, such as extreme temperatures. The spontaneous, in situ formation of cyclic dipeptides in aqueous buffer from a variety of dipeptide methyl esters, through intramolecular amide bond formation is demonstrated in chapter 4 resulting nanoscale morphologies that are dictated by amino acid side chain functionality, which is presented on the nanostructure surface. The approach provides a straightforward means of producing supramolecular architectures with tunable nanoscale morphologies and surface chemical properties. The formation kinetics and consequent supramolecular properties of the resulting structures can be regulated by simply varying the concentration of starting materials, as demonstrated for a nanostructured hydrogel with tunable stiffness. Moreover, when the co-assembly between a functional cationic metalloporphyrin molecule and cyclic dipeptide hydrogels is achieved by introducing the functional porphyrin in the starting mixture it can lead to non-covalent functionalization and formation of supramolecular structures with customizable peroxidase-like activity.

Unprotected tri-peptides even with the presence of three amino acids exhibit 8000 different combinations providing a large sequence space to study. In chapter 5 we show that supramolecular structures formed thorough cationic tri-peptide self-assembly can utilized to serve as a supramolecular platform to organize charged porphyrin molecules in different arrangements depending on the primary sequence of the peptide. The differences in spatial organization of
porphyrin molecules can be demonstrated by investigating the ability of the peptide-porphyrin assemblies to participate in energy transfer. The calculated energy transfer efficiencies will describe the degree of order of the porphyrin molecules inside the peptide supramolecular structures, thus enabling the controlled positioning of porphyrin molecules.
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1. Introduction

1.1. Introduction to Thesis

Peptides, the shorter form of proteins, exhibit similar biocompatibility and diversity to proteins. They provide a better accessibility in large scale with great stability and robustness. Additionally, specially designed peptides have shown the same function as proteins when the functional sequence is retained in the peptide. Thus, peptide nanotechnology is becoming an attractive field of study among the supramolecular chemistry research community due to its versatility. Nanostructures generated via self-assembly of peptides have served as smart materials in various applications such as electronics, food and cosmetics. These peptide-based nanomaterials display unique properties that arise from their primary building blocks, the 20 gene coded amino acids. The peptide sequence can be functionalized according our choice based on the amino acid residues, hence providing the opportunity to generated programmable primary structures for self-assembly via non-covalent interactions. Short peptides and their derivatives, especially di- or tri- peptides are of particular interest in this context due to the simplicity and availability.

Peptides are widely used as a scaffold for organizing chromophores, where the alignment of chromophores is directed by peptide assemblies. Porphyrins are one of chromophores that are well-studies as a monomer due to various applications associated with it. Peptide-porphyrin co-assembled nanostructures generated via non-covalent interactions will have advantages compared to traditional peptide-porphyrin conjugates. Generation of well-ordered peptide-porphyrin supramolecular structures will enhance functions of porphyrin such as light harvesting, catalysis and photosensitizing through flexible connections arising from soft-interactions and diversity of structures.
The ultimate goal of the projects presented in this thesis is to understand the self-assembly of linear and cyclic peptides and peptide modulated co-assembly of porphin in fabricating minimalistic designs of complex biomaterials using short peptides or peptide derivatives. Thermodynamic or kinetic self-assembly of linear, cyclic and amphiphilic peptides and co-assembly of porphyrin with peptides will be studied and presented in the thesis.

1.2. Thesis Outline

This thesis consists of five chapters. A literature review on short peptide self-assembly focusing on di- and tripeptides and aromatic amphiphilic peptides as assembly building blocks and peptide mediated porphyrin co-assembly is presented first (Chapter 2) followed by three research projects included in publication format (Chapters 3, 4 and 5). Each chapter will consist of an introduction, the objective of the project followed by results and discussion. Then the conclusion and materials and methods are presented before the references. Final part of the thesis will cover the conclusions derived from complete thesis and future directions (Chapter 6).

The biocatalytic co-assembly of a nanofibrous Fmoc protected di-peptide with porphyrin derivative in aqueous medium to generate an energy transfer hydrogel is presented in Chapter 3. This project demonstrated that depending on the concentrations of porphyrin used, the resulting nanofibrous gels show two distinct regions of self-assembly behavior. The cooperative assembly with porphyrin incorporation observed up to a critical concentration of porphyrin, and separate porphyrin self-aggregation observed at higher concentrations, hence facilitating the organization of porphyrin inside the peptide scaffolds depending on their concentration.
A straightforward and versatile way to produce peptide-based nanomaterials with customizable properties through the spontaneous aminolytic cyclisation and self-assembly of corresponding dipeptide methyl esters in aqueous media is described in Chapter 4. The chemical nature of the amino acid side chains dictates the supramolecular arrangement and resulting nanoscale architecture. Moreover, by adding functional components, exemplified by porphyrins, to the starting dipeptide ester solution, these become incorporated, which gives rise to the formation of nanofibers able to catalyze the oxidation of organic phenols in water.

Formation of sequence depending tri-peptide porphyrin co-assembled nanostructures is demonstrated in Chapter 5. The project was carried out to investigate the propensity of a cationic tripeptides to co-assemble charged porphyrins (positive and negative) inside the peptide matrix depending on the primary sequence of the peptide. The system shows that the organization of porphyrin molecules into the peptide assembles can be controlled by the sequence of the peptide. The calculated FRET efficiencies demonstrated that the supramolecular organization required to facilitate FRET can be obtained by tuning the peptide sequence and charge of the porphyrin used.

Finally, Chapter 6 will provide a summary of important findings derived from the thesis and potential directions towards future research in the field.
2. Literature Review: Peptide self-assembly and Peptide-porphyrin co-assembly
2.1 Self-Assembly

Self-assembly is defined as the process of the spontaneous organization of small molecular units into stable and ordered structures interacted via non-covalent bonds.\(^1\) In order to better understand the self-assembly systems, designer building blocks with specific assembly properties are required. These systems can be dynamic, reversible and provide tunability of the resulting structures with ranging applications in biology, medicine, food industry and nanotechnology. The work described on this thesis will be on bottom-up self-assembly approach using peptides and focussed on applications as in light harvesting and catalysis.

Various kinds of synthetic and biomolecules are serving as self-assembling motifs to form functional materials. Proteins, peptides and lipids are known buliding blocks that can be use to engineer new supramolecular structures of particular interest in biomaterials chemistry.\(^2\-^3\) Peptides and peptide amphiphiles have become one of the most widely used self-assembling buliding blocks due to their versatility and chemical richness.\(^4\)

2.2 Peptides as Self-Assembling Building Block

The Biocompatibility of peptides arises from their primary amino acid building blocks makes them a versatile platform for many applications. 20 gene coded amino acids offer diverse chemical functionality into the peptide side chain, amino acids are clarified as aromatic (Phenylalanine (F), Tyrosine (Y), Tryptophan (W), Histidine (H)), basic (Lysine (K), Arginine (R)), polar (Threonine (T), Serine (S), Glycine (G), Asparagine (N), Glutamine (Q)), aliphatic (Leucine (L), Isoleucine (I), Valine (V), Methionine (M), Alanine (A)), acidic (Aspartic acid (D), Glutamic acid (E)) and special (Glycine (G), Proline (P)) residues (Figure 2.1).
Programming or design of the primary structure of peptides following proteins but in a simple format with different combinations of amino acids enables the generation of customizable self-assembling motifs. As a class of supramolecular building blocks, organization of peptide building blocks into ordered structures can be facilitated by non-covalent interactions through the side chains of different classes of amino acids such as electrostatic, hydrophobic interactions, Van der Waals interactions, hydrogen bonding and π-π stacking interactions.

Self-assembly of shorter oligopeptides (2-20 amino acids) are of particular interest in this context due to their low structural complexity compared to proteins and large peptides. Since the first reports by Ghadiri et al. and Zhang et al. minimalistic peptide-based nanostructures have broadly

Figure 2.1: Primary building of peptides, 20 gene coded amino acids represented in single letter and color-coded spheres depending on their functionality. (This color-coded representation will be used to define the amino acid sequences in the thesis).
studied by many research groups leading to a wide variety of applications. Supramolecular chemistry of the shortest peptides (consists of 2-5 amino acids) has gained the popularity over past few years particularly as minimalistic nanomaterials generating diverse nanoscale architectures such as fibers, tubes, spheres, tapes and rods. Design and discovery of short peptide based supramolecular structures is typically based on either simplifying the naturally occurring designs based on prior knowledge, or through synthetic modification of peptides with aromatic or aliphatic amphiphilic ligands to enhance the self-assembling propensity. This part of the introduction mainly focused on the recent studies on self-assembling short oligopeptides and their applications.

2.2.1 Aromatic peptide amphiphiles

Small molecule self-assembling building blocks consisting of short peptides generally require a hydrophobic functional group to trigger the self-assembly via strong hydrophobic interactions. In this context, either aliphatic or aromatic peptide derivatives are of interest. Aliphatic peptide amphiphiles has been extensively studied but focus here will be on aromatic peptide amphiphiles. An aromatic amphiphilic peptide consists of four distinct units namely (i), aromatic moiety (ii), peptide component (iii), linker segment and (iv), C terminus. (Figure 2.2a.) The peptide component usually contains a short peptide sequence which is generally one to three amino acids and is modified at the N-terminus using an aromatic functional group connected through the linker. The C-terminus is also an important in self-assembly which can be functionalized (or capped) or kept unprotected to adjust the charge balance. Various types of aromatic moieties have been utilized in functionalizing the N-terminus and some typical designs are summarized in Figure 2.2a. Along with the aromatic moiety, the peptide sequence also plays an important part
in generating supramolecular structures through a balance of electrostatic forces and hydrogen bonding interactions.\textsuperscript{16,18} 

Among the various amphiphilic moieties reported Fmoc (9-fluorenylmethoxycarbonyl) is the first and still the most widely used synthetic moiety coupled to a short peptide to form supramolecular structures. This is due to availability of N-terminus protected Fmoc based amino acids which are utilize in solid phase peptide synthesis. The first report on Fmoc based hydrogelator was by Vegners et al.\textsuperscript{19} demonstrates a thermoreversible gel formed with Fmoc-LD through heat-cool cycles. the first report from Vegners et al.\textsuperscript{19} Nanostructures generated through Fmoc based peptide self-assembly have shown the propensity to be utilized in various applications.\textsuperscript{20} Gazit and co-workers examined Fmoc based peptide Fmoc-FF in generating an extremely stable hydrogel in water.\textsuperscript{21} Simultaneously, Ulijn group has reported a self-supporting hydrogel of Fmoc-FF at physiological pH in aqueous conditions that is stable under cell culture conditions.\textsuperscript{22} Starting from
2004, a large number of self-assembly studies on amphiphilic peptides have published with different aromatic synthetic moieties showing a steady growth in the field. Beyond Fmoc, they include aromatic moieties such as naphthalene, pyrene, azobenzene, phenyl, perylene and many others (Figure 2.2.a).\textsuperscript{10-13}

The aromatic derivative on the N-terminus plays a vital role in self-assembly and the formation of hydrogels. Fmoc derivatized short peptides have shown the ability to facilitate consistent hydrogelation compared to most hydrophobic non-aromatic groups for instance Boc(tert-butoxycarbonyl).\textsuperscript{23} It is also reported that pyrene groups can trigger hydrogelation with less hydrophobic peptide sequences such as AA compared to more hydrophobic FF.\textsuperscript{10} Furthermore, naphthalene has utilized as a hydrogel forming synthetic aromatic moiety with less hydrophobic peptide sequences as VYGGG.\textsuperscript{24} In addition to stable hydrogel formation various other applications such as energy transfer and charge transfer supramolecular systems has also reported by exchanging the aromatic moieties for functional (semi conducting) moieties.\textsuperscript{25-26}

The peptide sequence linked to the aromatic moiety is a another crucial factor in generating the self-assembled nanostructures. The hydrophobic amino acids specifically aromatic residues (F, Y, W) have shown a higher aggregation potential compared to hydrophilic residues. It is reported that the hydrophilic and charged amino acid residues (S, T, Q, N, E, K) also can lead to hydrogel formation when combined with aromatic amino acid residues.\textsuperscript{27}

Along with the suitable aromatic amphiphilic moiety and peptide sequence, the self-assembly route is also of greater importance.\textsuperscript{28} Thermodynamically controlled, kinetically routed or non-equilibrium driven self-assembling approaches in the design of supramolecular structures are described in literature in controlling the structure formation. In this literature review the work based on Fmoc peptide self-assembled nanostructures based on short peptides and aromatic
amphiphilic peptides and their respective applications will be examined using thermodynamically controlled self-assembling pathway triggered by an environmental change (solvent polarity, temperature) or enzymatic reaction.

2.2.2. Non-covalent interactions governing aromatic amphiphilic peptide self-assembly

The important non-covalent interactions that govern the self-assembly of peptide building blocks have been mentioned in an earlier part (section 2.2) of the literature review specific to aromatic amphiphilic peptides, self-assembly is directed by aligning the relatively hydrophobic and hydrophilic regions of the molecule through hydrogen bonding and π-stacking. A number of elementary stacking arrangements are of greater importance in stabilizing the supramolecular structures based on the interactions. The aromatic amphiphile facilitate the π-π stacking interactions depending on the organization of aromatic moieties. They can be categorized into parallel, anti-parallel or interlocking anti-parallel according to their arrangement presented in the nanostructures. Smith et al. describes the hydrogel formation through π-π interlocked β-sheets using Fmoc-FF. In their model the interlocking Fmoc moieties of alternate β-sheets generate π stacked pairs positioning them in such a way that they interact via the phenyl ring (Figure 2.3).

**Figure 2.3:** a). anti-parallel β-sheets creating the interlocking of Fmoc groups to form π -stacked pairs (b). rotated second sheet formation due to twisted β-sheets to maintain Fmoc interaction. fluorenyl groups: orange and the phenyl groups: purple. Adopted from ref. 29
Hydrogen bonding between the peptide residues can also induce the β-sheet formation. Generation of a β-sheet type organization in the supramolecular structure plays a significant role in facilitating the hydrogelation. Depending on the balance between hydrogen bonding and π-stacking they can generated morphologies such as spheres, worms, sheets tapes, fibers and tubes.\textsuperscript{7}

2.3 Biocatalytic Self-Assembly of Aromatic Amphiphilic Peptides

Biocatalytically triggered self-assembly is one of the well-studied strategies to induce the supramolecular structure formation.\textsuperscript{30} The self-assembly triggered by enzymatic catalysis is inspired by the biological systems where the assembly and disassembly is prompted by biocatalysts such as enzymes. Thermodynamically controlled assembly can provide the direct correlation between supramolecular order and function. Traditional supramolecular systems are mainly routed via thermodynamically controlled pathway to generate most stable thermodynamic structure. Most of the biological examples consisting of supramolecular structures such as protein folding and

![Diagram](image)

**Figure 2.4:** Thermodynamically controlled self-assembly forming the most stable structure through free energy landscape
assembly, liposome formation and transcription of nucleic acids are controlled through thermodynamics. In these equilibrium driven systems the supramolecular structures formed are the most stable ones formed representing a global minimum in the free energy landscape.

Enzyme catalyzed hydrolysis/condensation reactions are known to favor the amide bond breaking rather than favoring the formation of the bond in aqueous media. But the stable supramolecular structures produced can shift the equilibrium towards bond formation as the free energy of hydrolysis reaction ($\Delta G_{\text{amide hydrolysis}}^0 = -4$ KJ/mol) also close to equilibrium. This approach is fully reversible and allows the structural corrections during the self-assembly process providing the thermodynamically most stable structure (Figure 2.4).

The first report on biocatalytic self-assembly under thermodynamic control was by Toledano et al., in 2006 from our group. A reverse amide condensation/hydrolysis reaction was performed using a non-specific endoprotease (thermolysin from Bacillus Thermoproteolyticus rokko) between a series of inactive gelation precursors. A series of Fmoc appended amino acids (Gly (G), Ala (A), Val (V), Leu (L), Pro (P), Phe (F)) was enzymatically coupled with a dipeptide (F$_2$ and L$_2$) to form the amide bond under thermodynamic control to generate a self-assembling Fmoc-tripeptide. It was demonstrated that the self-assembly propensity was higher when there is a higher yield in amide bond formation where dipeptides F$_2$ and L$_2$ exhibited an organization into a fibrillar network compared to other amino acids. (Figure 2.5).
This was followed by a systematic study on biocatalytic self-assembly on a series of non-assembling precursors demonstrating the fully reversible enzymatic condensation reaction allowed for component selection and supramolecular structural correction. Component selection was explored with a library of Fmoc amino acids (Gly (G), Leu (L), Phe (F), Thr (T)) coupled to a set of nucleophilic dipeptides (G2, F2 and L2) or amino acid esters (L-, F-OMe) in the presence of thermolysin. The concept was based on Dynamic Combinatorial Libraries (DCL), where multiple

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**Figure 2.5:** a). Thermolysin catalyzed reaction between Fmoc appended amino acid and a dipeptide b). chemical structures of amino acid side chains for R1 c). hydrogel formation after addition of thermolysin d). cryo SEM image of nanofibers formed e). percent yield of amide bond with different amino acids. Adopted from ref. 31
components are reversibly combined and exchanged to generate the most stable structure with lowest free energy. This concept was demonstrated for example with Fmoc-F and F₂ utilizing time dependent HPLC, where it was observed that the thermodynamically most stable structure is formed over time competing with kinetically stable structures demonstrated by Fmoc-F coupled with F₂ ultimately giving rise to Fmoc-F₃ and Fmoc-L coupled with L generating Fmoc-L₅. Conclusively, this work described the first example of using biocatalytic self-assembly to trigger dynamic component amplification in a DCL concept. (Figure 2.6.)

The enzyme catalyzed condensation of amino acids has also been utilized to determine the sequence/structure relationship of Fmoc appended di-peptide hydrogels. The authors have investigated the self-assembly of four closely related Fmoc di-peptide methyl esters, Fmoc–SF–OMe (SF), Fmoc–SL–OMe (SL), Fmoc–TF–OMe (TF) and Fmoc–TL–OMe (TL) formed using the precursors Fmoc–T, Fmoc–S, F–OMe and L–OMe with thermolysin as the catalytic trigger. Although, all four systems showed the β-sheet formation interlocked through extended π-π stacking interactions of Fmoc moieties, they showed significant differences in supramolecular
Figure 2.6: a). Reversible biocatalytic reaction between Fmoc-amino acid and dipeptide/dipeptide ester b). gel formation over time and c). TEM images of Fmoc-F and F₂ over time after addition of thermolysin time dependent HPLC conversions for formation of most stable structures with the reaction between d). Fmoc-F and F₂ and Fmoc-L and L₂ f). conversion of fibers to sheet like structure after addition of thermolysin to Fmoc-L and L₂ imaged by AFM. Adopted from ref. 30

organization. Transmission Electron Microscopy (TEM) analysis has revealed that different nanoscale morphologies such as sheets, belts, short and long fibers have observed in the hydrogels
formed. It was also reported that the strength of the hydrogels significantly changes with the amino acid sequence in the peptide (Figure 2.7.).

A computational approach was used to investigate the molecular interactions within an Fmoc-peptide based supramolecular system (Fmoc-TF-NH₂) for better understanding of H-bonding and aromatic interactions. The reversible nature of the biocatalytic self-assembly system ensured that the thermodynamically optimized structure was formed, thus allowing direct connection between molecular dynamics simulations and experiments. The authors demonstrate the validation of developed atomistic models by MD simulations by assessing their relative stabilities (Figure 2.8).
Taking the experimental data into consideration, two models were built in an anti-parallel arrangement in such a way that they maximize the interactions. The TEM images suggested that supramolecular structures form fiber like and twisted ribbon like morphologies, therefore a two-fiber model and a bilayer model were constructed for the simulation to compare their stabilities. The simulated models over time suggested the structure formed after self-assembly consists of laterally aggregated fibers via H-bonding of Threonine (T) side chains with carbamate oxygen of Fmoc groups. This confirms the model suggested for Fmoc-F which was discussed in section 2.2.2.\textsuperscript{29} Furthermore, the T-F interactions are also involved in H-bonding through F backbone with amidated C-terminus of T.

\textbf{Figure 2.8}: (a) 2D and (b) 3D-vdw representations of Fmoc-TF-NH\textsubscript{2} c). side view of fiber model d). H-bonded conformation with 2.1 corresponding to Fmoc–F, 2.2 to F–F, and 2.3 to T–F e). cryo TEM f). TEM images of Fmoc-TF-NH\textsubscript{2} . Adopted from ref. 33
Figure 2.9: a). Thermolysin catalyzed fully reversible condensation reaction and donor molecule dansyl-β-alanine (DA) b). percent HPLC conversions monitored overtime time in the absence (solid traces) and presence (dashed traces) of DA c-d). AFM images of DCL and DA before and after addition of thermolysin e-h). TEM images of DCL with DA and individual components (e). before and (f-h). after addition of thermolysin, (f). without (g). with DA and (h). isolated YF and DA i). fluorescence measurements showing enhanced energy transfer in DCL system before (solid traces) and after (dashed traces) the addition of thermolysin (j). isolated YF system before (solid traces) and after (dashed traces) the addition of thermolysin at variable concentrations of DA. Adopted from ref. 24
Thermodynamically driven self-assembly, furthermore showed the potential to generate functional nanomaterial such as energy transfer\textsuperscript{25} and charge transfer nanostructures.\textsuperscript{32} Design and characterization of efficient energy transfer hydrogels utilizing DCL approach with naphthoxy appended di-peptide library as a donor and dansyl-β-alanine (DA) as an acceptor has reported.\textsuperscript{26} The enzyme (thermolysin) catalyzed amide bond formation has led to formation of optimized supramolecular structures with rapid and efficient energy transfer. Thus, the DCL strategy allows the generation of thermodynamically most stable conformation providing enhanced functionality without unwanted kinetic aggregates that retard the performance (Figure 2.9.).

2.4. Unprotected Short Peptides

Apart from the aromatic peptide amphiphiles, a widely employed self-assembling strategy is to use the unprotected peptides as the building blocks. Short peptide with di- and tri- peptides are of interest of in this context due to their simplicity, showing the ability to generate minimalistic versions of more complex biological systems.\textsuperscript{34-35} Although the simple di- and tri- peptides are consisting of just two or three amino acids, they provide a wide range of sequence space having 400 and 8000 combinations respectively, giving rise to different structures.\textsuperscript{36-38} In this section, peptide self-assembly with short linear peptides (di- and tri-peptides) and cyclic peptides will be discussed in detail.
2.4.1 Linear Peptide Self-Assembly

2.4.1.1. Di-peptide self-assembly

Di-peptides as the simplest peptide available consists of two amino acids in the side chain can make 400 different combinations available with 20 amino acids present. The first systematic study on supramolecular chemistry of short unprotected peptides with tunable sequences (LL, LF, FL and FF) was reported by Görbitz. Their paper contained the single crystal X-ray diffraction analysis obtained by crystallization of aqueous solutions of peptides. One of the first studies on self-assembly of nanostructures from short peptides was also on di-peptide namely di-phenylalanine (FF), a motif known to form supramolecular structures in β-amyloid polypeptide. In this work, the supramolecular chemistry of FF in aqueous medium was investigated and well-ordered, tube-like nanostructures were formed upon rapid dilution of high concentrated FF in 1,1,1,3,3,3 hexafluoro 2-propanol via H-bonding between the amino acid residues in peptide backbone and π-π stacking interactions between aromatic groups (Figure 2.10.).
Among the assembling di-peptide sequences reported, phenylalanine (F) containing dipeptides have obtained a greater interest and most studied self-assembling motifs forming various kind of nanoarchitectures depending on the sequence and self-assembly pathway.\textsuperscript{41} Since its original discovery, FF has shown to form tubular nanostructures of high thermal and chemical rigidity with metal-like stability, piezoelectric, semiconducting and optical properties and vesicular structures at lower p\textsc{hs}.\textsuperscript{34, 42} Apart from FF, different di-peptides have been reported to assemble into supramolecular architectures. For example, FW forms tubes; WW and WY form disordered aggregates; IF forms a fibrous hydrogel while VF is not aggregating to form any structures, explaining the importance of sequence.\textsuperscript{35-36} The supramolecular aggregation potential of entire di-

\textbf{Figure 2.11:}  a). AP score for dipeptide represented by two-dimensional grid b). MD simulations of FF. Red: backbone beads, white: side chain beads. Without water beads (i) 0 \textmu s; randomly placed dipeptides in the periodic box and formation of (ii) 0.2 \textmu s sheet-like aggregates (iii) 0.5 \textmu s vesicles by sheet folding. (iv) 1.5 \textmu s a hollow tube by fused vesicles forming a hollow tube (blue bead: water) Adopted from ref. 43

peptide sequence space of 400 (20\textsuperscript{2}) peptides has investigated using coarse-grained molecular dynamics simulations to explore the di-peptide with potential to generate supramolecular structures.\textsuperscript{43} The results indicated that the introduction of charged amino acid residues has a direct
effect on reducing the self-assembling propensity while a combination of an aromatic residue followed by an aliphatic residue enhances the formation of supramolecular aggregates (Figure 2.11.).

A searchable dynamic peptide library (DPL) approach has been reported based biocatalytic self-assembly under thermodynamic control as discussed in section 2.3. A set of homo- and hetero-dipeptides including aromatic, aliphatic, polar and charged amino acids were explore to generated the most stable peptide component through continuous enzymatic condensation, hydrolysis and sequence exchange. Homo- and hetero-dipeptides composed of F, L, W, S and D, which were subjected to enzymatic condensation/and over time they formed assemblies exhibiting a range of interesting morphologies. Among the homopeptides, F_2 to F_6 demonstrated a time dependent conversion of tubular structures to short nanofibers (Figure 2.12. b), L_6 exhibited tape-like morphology (Figure 2.12. c) and spherical aggregates were formed with W_4 (Figure 2.12. e). The heteropeptide FDFSFDFS was selected as the dominant peptide from FS/FD dipeptides and was able to generate fibrous nanostructures over time competing with other possible sequences obtained (Figure 2.12. e).
Tri-peptides have 8000 possible combinations amino acids. There are a few previous reports on tripeptide self-assembly in aqueous medium forming various types of nanoarchitectures. Most reports are based on the tripeptide containing di-phenylalanine with one additional amino acid. FFF is known to form fibers and plate-like nanostructures, CFF forms nanospheres. Changing the chirality from non-assembling L-derivative to D-derivative in N-terminus of DVF and DFFV forms nanotapes and nanofibers respectively. The D-derivatized DLFF formed more entangled ordered 3D fiber network compared to L isomer. Furthermore, it is has been shown that introducing the D as the middle residue in FDLF have also forms a self-standing hydrogel with thicker fibers compared to DLF. KFG has been reported to switch between micelles and nanotubes by lowering pH. The above studies indicate that most of the tripeptide sequences that
have shown the ability to self-assemble were discovered by chance or by systematically changing the known sequences.

To investigate the potential candidates from entire tripeptide sequence space to form self-assembled structures, the design rules on the sequence order of all 8000 possible tripeptide combinations were computationally screened by Tuttle, Ulijn and co-workers. The study was based on calculating the aggregation potential of tripeptide sequences by obtaining the ratio of Solvent Accessible Surface Area (SASA) before and after self-assembly using a course-grained model (Figure 2.13). A total hydrophobicity factor (log P) was introduced to explore the aggregation potential of not only highly hydrophobic but also the weakly hydrophobic sequences. The simulation results revealed that the position of certain amino acids is critical for aggregation. The authors have discovered that the aromatic amino acids (especially F and Y) found in the middle or C-terminus of the sequence showed a higher aggregation potential than when found in N-terminus.
Figure 2.13: a). Heat maps generated for the normalized score of 8000 tripeptide combinations after 50 s simulation b). magnified view of highlighted area c). average AP$_H$ scores of tripeptides depending on their position TEM and photographic images of d). KYF e). KFD and f). PFF . Adopted from ref. 50
While the positively charged amino acids (K and R) were preferred in the N-terminus, negatively charged amino acids are ideal in the C-terminus for enhanced aggregation. The H bond donating amino acids (S, T, Q and N) presented in the N-terminus support also favor the aggregate formation. After validating the computational results by experiments they have reported four hydrogel forming tripeptides namely KYF, KFF, KYY and KYW that self-assembled into fibrous

**Figure 2.14:** a). Chemical structures of tripeptides and schematic diagram of self-assembly and polymerization b). Photographic images of self-assembled peptides and tyrosine c) TEM analysis of structures formed after self-assembly d) preferred confirmation of each peptide b). polymeric peptide pigment structures by TEM. Adopted from ref. 51
nanostructures upon adjusting the pH to 7.5 in water. These were the first example of tri-peptide hydrogels that formed in completely aqueous medium.

The studies on anionic tripeptides have extended to investigate the ability to act as a tunable precursor for polymeric pigments such as melanin. A set of tyrosine containing anionic tripeptide-amides (FDY, YDF, FYD, YFD, DFY and DYF) was examined to explore the potential to form supramolecular nanostructures. The self-assembled structures of tripeptides showed different morphologies depending on their primary sequence. It was observed that sequences with adjacent aromatic residues (FYD, YFD, DFY, DYF) form ordered nanostructures while no assembly was observed for sequences with unpaired aromatic residues (FDY and YDF) (Figure 2.14. b and c.).

Computational models and experimental validations have utilized to further understand tri-peptide self-assembly. Selective complex formation of co-assembled tripeptide has demonstrated utilizing a structure forming tripeptides (FFD and DFF) and a Cu$^{2+}$ binding GHK for the co-assembly. GHK is known to be a non-gelator while FFD and DFF form bi-layer like structure but no hydrogelation at neutral pH. Equimolar mixtures of FFD and GHK had generated tape-like structure, which had spontaneously transformed into a hydrogel upon addition of an equimolar solution of CuCl$_2$ resembling the color of CuCl$_2$ confirming the selective complexation of co-assembled peptides towards Cu$^{2+}$ (Figure 2.15 d. and e.).
Formation of stable emulsions in oil/water interface have reported including KYF, KFF, KYW, DFF, FFD (Figure 2.16, a-c). The peptides of interest have shown a difference in stability of emulsions formed depending on the sequence and temperature. Fibrous nanostructures were obtained from cationic tripeptide, KYF, KFF and KYW while the anionic tripeptides (DFF and FFD) exhibited a formation of bilayer-like structures. These cationic and anionic peptides showed two different emulsification behaviors. The oil droplet formation was stabilized by KYF, KFF and KYW using the nanofiber network and DFF and FFD displayed properties of traditional surfactant by self-assembling in oil.

**Figure 2.15:** a). Chemical Structures of tripeptides and schematic diagram of fibers formed at oil-water interface b). photographic images of supramolecular structures in the following order KYF, KFF, KYW, DFF, FFD (left in water, right oil/water emulsion) c). Computational simulations frame at 9.6 μs biphasic systems d). TEM images of tripeptides (i) GHK (random aggregates) (ii) self-assembled FFD (nanofibers) (iii) co-assembled FFD/GHK (nanofibers) (iv) co-assembled FFD/GHK complexed with copper ions and the insects shows the photographic images structures formed e). Computational analysis of self-assembling tripeptides indicating co-assembled nanostructures at ~9.6 μs. Adopted from ref. 52,53
Biocatalytic self-assembly by phosphatase catalyzed de-phosphorylation of unprotected tripeptides in both aqueous and biphasic media have also studied. It could be demonstrated that the enzyme catalysis (alkaline phosphatase) utilized in self-assembly exhibits a direct effect on the morphology of nanofiber network formed and oil-in-water emulsion stabilization by forming the nanofibers both in organic/water interface and surrounding aqueous environment (Figure 2.16).

Physicochemical and biological characterization of tripeptide (KYF) metal (Pt) nanoemulsion consisting of oleic acids – Pt(II) conjugated core (KYF-Pt-NE) have been published. The authors concluded that the physicochemical properties and efficacy of the nanostructures formed have the potential to function as a biodegradable drug carrier suggesting to vary properties of the scaffolds by introducing new peptides in future (Figure 2.17).
**Figure 2.17:** a). Structure of Oleic acid-Pt (II) conjugate b). KYF tripeptide-platinum (II) nanoemulsion (KYFPt-NE) (left) and TEM image of nanoemulsion formed (right) b). (i) photographic images (ii) stability tests, of Pt-NE and KYF-Pt-NE in water (iii) Pt (II) release from KYF-PtNE in vitro at different pH (in PBS). Adopted from ref. 55

**Figure 2.18:** a). Structure of L\textsuperscript{D}P\textsuperscript{D}F tripeptide and oxidized nanocarbons b). TEM micrographs of nanocarbon incorporated peptide hydrogels (i) with CNT, (iii) with GO and (v) with CNH c). stress recovery potential of hydrogels in the presence of each nanocarbon compared to the peptide. Adopted from ref. 56
Further studies on the effect of chirality in amino acid residues on supramolecular structure formation was investigated by introducing the D-enantiomer amino acid into the peptide side chain.\textsuperscript{56} The authors have shown that the addition of oxidized nanocarbon material such as carbon nanotubes as 1D structure (CNT), graphene oxide sheets as 2D structure (GO), and carbon nanohorns as 3D structure (CNH) into the Leu-DPhe-DPhe peptide hydrogel matrix can enhance the mechanical properties (Figure 2.18). The rheological stress recovery studies on peptide-nanocarbon hydrogels has revealed that the CNT incorporated hydrogels have gained extra strength showing a better self-healing potential.

2.4.2. Cyclic Peptide Self-Assembly

Apart from the linear peptides cyclic peptides have also gained the attention as a versatile molecular building block for self-assembly. Due to their unique physical and chemical properties such as enzymatic stability and molecular rigidity arising from its structure, cyclic peptides were utilized in designing functional materials in recent years. The first example on cyclic peptide self-assembly was demonstrated by Ghadiri in 1993 with a eight residue cyclic peptide sequence namely cyclo[-(D-Ala-Glu-D-Ala-Gln)\textsubscript{2}] containing alternate stereochemistry (D, L) leading to formation of tubular nanostructures.\textsuperscript{57} They have further extended the studies to propose antimicrobial and antiviral properties as well as potential use as designed membrane proteins by tuning properties with amino acid side chains presented to the exterior of the nanotube.\textsuperscript{58-59} Furthermore, charge transfer nanotubes are reported with cyclic D,L-\textalpha- peptide appended with 1,4,5,8-naphthalenetetracarboxylic acid diimide.\textsuperscript{60} After the first few examples of self-assembling cyclic peptides and their respective applications, much shorter cyclic peptides have also reported, presenting amino acid functionality at the exterior of the nanostructure.\textsuperscript{61-67} This section of the
introduction will focus on self-assembly and their applications of short cyclic peptides with a focus on di-peptides.

Govindaraju and his co-workers have extensively studied the self-assembly of cyclic peptide derivatives especially in the context of short dipeptides.\textsuperscript{68-71} They have reported the spontaneous formation of fiber bundles using the most widely used aromatic peptide cyclic FF. Cyclic peptide was synthesized and then subjected to self-assembly in the presence of an organic medium such as chloroform with a trace of trifluoroacetic acid. The resulting fibers have generated fiber bundles forming a gel. (Figure 2.19, a-c).\textsuperscript{72}

\textbf{Figure 2.19}: a). chemical structure of cyclic FF and hydrogen bonding in supramolecular structures, FESEM images of b). fiber bundles and c). fibers on xerogel, d). chemical structures of unnatural cyclic peptides HRTEM images of e). 2D meso sheets f). nanosheets in CHCl\textsubscript{3}-TFA formed by pure L-derivative g). crystalline rhomboid sheets obtained from L-D derivative. Adopted from ref. 72,73
The group have further extended the self-assembling study on an unnatural cyclic peptide, cyclo(Phg-Phg) and cyclo(L-Phg-D-Phg). Initially the cyclo(Phg-Phg) form 2D nanosheets. Then these nanosheets self-organize into 2D mesosheets through intermolecular hydrogen bonding resembling the natural materials with layered architecture. The respective L and D stereoisomer of the peptide cyclo(L-Phg-D-Phg) self-assembled into non-crystalline and single crystalline 2D sheets exhibiting large lateral dimensions highlighting the large scale 2D sheet production utilizing one of the simplest unnatural aromatic cyclic peptides (Figure 2.19. d-j).^73

**Figure 2.20:** a). cyclization of dipeptide FF by vapour deposition and self-assembling scheme from linear peptide spheres to cyclic nanotubes b). top c). side SEM images of nanotubes d). high water contact angle and e). patterned microfluidic devices using nanotubes f). solvothermal synthesis of cyclic FF and self-assembly into ultralong nanobelts g). evolution of nanostructures at different temperatures and h). bright field (left), fluoresce (middle) images of NR-loaded crystalline platelets f). optical waveguiding cross the platelet. Adopted from ref.
Formation of tubular nanostructures from cyclic FF was also studied using chemical vapor deposition by Gazit’s group. Linear dipeptide FF was subjected to evaporation at 220 °C inside a vacuum chamber. The cyclization of linear peptide and self-assembly of cyclic peptide take place during this process. Nanotubes obtained by self-assembly under chemical vapor deposition were several micrometers in length and 50-300 nm in length. They have fabricated aromatic di-peptide nanotube coating on glass substrates, resulting in hydrophobic modified surfaces. Using the standard vapor deposition technique, they have utilized the FF nanotubes modified surfaces in designing micro fluidic chip on a silicon substrate having a control over capillary flow speed (Figure 2.20 a-e). Other reports on cyclic FF demonstrated the production of crystalline
nanobelts using a solvothermal approach by resulting hierarchically oriented crystallization of cyclic FF.\textsuperscript{64, 75} The cyclic peptide gels prepared in 1,1,1,3,3,3-hexafluoro-2-propanol/toluene mixture were heated in a temperature ramp until the gel collapses and solution starts to boil producing nanobelts with micrometer width and length and nanometer height. Further heating produced rectangular ultralong nanobelts with ideal dimensions for optical waveguiding (Figure 2.20 f-h).\textsuperscript{64}

The effect of stereochemistry on supramolecular structure formation was studied with diastereoisomers of cyclic peptide cyclo(YA) (Figure 2.21). The L derivative (cyclo (\textsuperscript{L}-Y \textsuperscript{L}-A)) and D-derivative of Alanine (cyclo (\textsuperscript{L}-Y \textsuperscript{D}-A)). It was reported that the change in the chirality of alanine residue on the cyclic dipeptide has a direct impact on the supramolecular structure formation. The cyclo (\textsuperscript{L}-Y \textsuperscript{L}-A isomer self-assembled into nanotubes and nanowires while the cyclo (\textsuperscript{L}-Y \textsuperscript{D}-A) only formed microtubes. It has also revealed that the both peptides crystallize in the P21 space group and monoclinic system when the crystal and molecular structures were investigated by PXRD.\textsuperscript{76}
2.5 Peptide Mediated Porphyrin Co-assembly

2.5.1 Porphyrins

The porphyrins are stable aromatic macrocyclic compounds found naturally. They are vital in maintaining the metabolism of living organisms in the form of chlorophylls and hemoglobin.\textsuperscript{77}

The overall structure consists of a tetrapyrrole core which is bridged through methine groups that contains tetradeutate ligand at the center, thus creating a cavity that acts as a host for metal ions such as Fe\textsuperscript{3+}, Co\textsuperscript{2+}, Zn\textsuperscript{2+}, Mn\textsuperscript{2+}. Overall this forms a stable metal-ligand complex, namely metalloporphyrins (\textbf{Figure 2.22}).\textsuperscript{78} Porphyrins and metalloporphyrins have gained interest due to their availability as different derivatives and well-studied physical and chemical properties.\textsuperscript{79-81}

A variety of applications associated with porphyrins in its self-assembled state and/or co-assembled with a scaffold.\textsuperscript{82-84} They provide tremendous platforms for a variety of materials applications, including field responsive materials for optoelectronic applications, mimicking photosynthesis by photoinduced electron transfer and catalytic activities, biomedical applications in the context of photodynamic therapy.\textsuperscript{85-90}

\textbf{Figure 1.22:} Simplest free base porphyrin a). chemical structure b). ball and stick model
Although the porphyrins are being extensively studied as monomers or in their self-assembled state, organizing porphyrin molecules into supramolecular architectures using different scaffolds is still a challenge. Arranging functionalized porphyrin molecules with the aid of supramolecular platforms of DNA, proteins and peptides have been reported to enhance corresponding functionality of the porphyrin molecules.

Peptides and peptides derivatives including aromatic peptide amphiphiles are potentially used as a matrix for organizing porphyrin molecules to enhance their functional performances. Porphyrin molecules can be introduced into a peptide scaffold by means of peptide-porphyrin covalent conjugates or by exploring the non-covalent interactions between the peptides and porphyrin molecules. Having the molecules interact via non-covalent interactions has a few advantages over covalent connections. The rigid connections through covalent interactions limits the diversity of supramolecular structures form while non-rigid flexible connections of non-covalent interactions can lead to the formation various nanoarchitectures. Here we summarize recent advances in peptide mediated porphyrin co-assembly via non-covalent interactions with regards to few applications especially in the context of short peptides.

2.5.2 Material Applications of Porphyrin co-assembled Peptide Nanostructures

Development of biomimetic light harvesting systems is one of many applications of peptide-porphyrin self-assembled nanostructures. Photosynthesis and light harvesting takes place in green plants, algae and cyanobacteria, and makes use of an effective light harvesting systems to utilize the solar energy. Fabrication of artificial photosynthetic systems would ultimately allow for the production of chemical energy (fuels) from solar energy via photosynthesis like processes. In natural light harvesting systems, the light capture and transfer are accomplished by neatly aligned chromophores, namely chlorophylls. Optimized by billions of years of evolution, complex
systems of proteins maintain the demand for the precise and definite arrangement of chromophores for effective light harvesting in two different photosystems known as II and I. These facilitate the Excitation Energy Transfer (EET) from photosystem II to the reaction center at photosystem I. \textsuperscript{102-105} To achieve efficient EET, light harvesting systems prefer J aggregation (face to tail arrangement) to H aggregates (head to head arrangement) of chromophores. \textsuperscript{106-108} Highly ordered supramolecular assemblies are capable of providing well-organized orientation of chromophore to assist effective delocalization of photon energy absorbed by the chromophores thus supporting

\textbf{Figure 2.23}: a). Schematic diagram of self-assembled FF and coassembly of THPP b). Photocurrent measurements of FF-THPP on ITO electrode (dotted line) and ITO only (solid line) c). cyclic voltammograms of electron mediator only (M), FF/THPP with and without mediator Adopted from ref. 112
wave-like propagation of excitons.\textsuperscript{109-111} Hence, there is significant current interest in the design and synthesis of less complex synthetic systems that are inspired by natural photosynthesis, by taking advantage of supramolecular chemistry approaches to organize matter.

\textbf{Figure 2.24:} a). Interaction between Fmoc-FF and SnTPyP b). photographic image of hydrogel and (i) SEM (ii) TEM image of peptide-porphyrin fibers c) photocurrent measurement comparison d). generation of oxygen from solar water oxidation. Adopted from ref 115
One of the widespread strategies of arranging porphyrins is by means of peptides or peptide templates is via non-covalent interactions between self-assembled peptides and porphyrin molecules. Recent literature has demonstrated several approaches utilizing light harvesting.

**Figure 2.25:** a). Schematic representation of self-assembly and co-assembly b-c). Time dependent absorbance indicating the formation of peptide -porphyrin nanorods d). TEM image of aggregated nanorods and nanotubes e). comparison of absorbance intensity at 353 nm for tri-iodide formation Photocatalytic reduction of Pt$^{2+}$ on the surface on microspheres e) TEM image of higher magnification f). Photocatalytic reduction of 4-nitrophenol (4-NP) into 4-aminophenol (4-AP) by microspheres monitored by increase in peak intensity at 290 nm, corresponding to the production of 4-AP. Adopted from ref. 106
The co-assembly of FF nanotubes with Pt nanoparticle embedded meso-tetrakis(4-sulfonatophenyl)porphine (TPPS) produces nanostructures for light harvesting has studied as they exhibit the formation of J-aggregation upon self-assembly. The peptide-porphyrin system has shown that the Pt nanoparticle incorporated nanotubes can support electron separation leading to photocurrent responses when irradiated with visible light (Figure 2.23).

It was also observed that Fmoc appended dipeptide of phenylalanine (Fmoc-FF) self-assembled to form nano fibrous hydrogels with metalloporphyrin (meso-tetra(4-pyridyl)porphine (TPyP)) incorporation. This hydrogel is capable of producing the preferred oxidation potential for water splitting reaction generating H₂ and O₂ (Figure 2.24).

FF dipeptide has been further used to design photostable microspheres consists of peptide-porphyrin nanorods generated via hierarchical assembly of phenylalanine-phenylalanine FF and porphyrin directed by electrostatic interactions in an acidic pH (<2.0). In this work, sulphonated porphyrin (TPPS) was incorporated with FF considering their abilities to self-assemble forming J-aggregates at lower pH. The microspheres were composed of several photocatalytically active compartments consisting peptide-porphyrin nanorods thus facilitate catalysis in photocatalytic reactions (Figure 2.25).

The same principle of hierarchical self-assembly of peptide-porphyrin have been exploited furthermore, by changing the peptide sequence to KK with TPPS. The assemblies long fiber bundles with individual nanorods and nanofibers arranged along the bundles of fibers. It was evident that porphyrins are arranged into J-aggregates introducing anisotropic birefringence with enhanced helicity and photostability (Figure 2.26).
Figure 2.26: a). Schematic diagram of peptide mediated porphyrin self-assembly between KK/TPPS formation fibers bundles monitored by b). absorbance c). CD spectroscopy TEM images of e). fiber bundles e) fibers aligned in long range f-g). comparison of absorbance intensity at 353 nm for tri-iodide formation. Adopted from ref. 116
It was further reported that KK could be used for the co-assembly of TPPS in highly acidic medium (pH =2) at high temperature (70 °C) in the presence of mineral (Na⁺, Ti⁴⁺, Pt²⁺) containing water. They have reported the photocatalytic hydrogen evolution mimicking the probiotic metabolism and reactions in photosynthetic mechanism in photobacteria (Figure 2.27).\textsuperscript{117}

**Figure 2.27:** a). schematic representation of primitive photosystem b). TEM images of mineralized nanoparticles c). HRTEM images of TiO₂ and Pt nanoparticles on fibers d). (i)-(iii) hybrid fibers with N, Ti and Pt respectively e). Time dependent H₂ production. Adopted from ref. 117

Photodynamically reactive peptide-porphyrin based nanoparticles are reported using Fmoc-L₃-OMe and m-TPPS with enhanced anti-tumor treatment properties via solvent shifting method.\textsuperscript{118}

The authors have shown that the organization of porphyrin molecules inside the peptide scaffolds can be controlled by the molar ration between peptide and porphyrin. m-TPPS molecules were presented in their monomeric state with in the nanoparticles avoiding unwanted self-aggregation. The monomeric state of m-TCPP molecules which, was exposed on the surface of the
nanoparticles, was kept intact up to certain concentration facilitating the monomeric delivery of the molecule. The m-TCPP molecules on the surface were capable of achieving light induced $^1$O$_2$ production and diffusion at very low photosensitizer loading amount which minimized the side effects coming from excess uptake of PS (Figure 2.28).

**Figure 2.28**: a). Illustration of fabricating the nanoparticles b) SEM c). TEM images of peptide-porphyrin nanoparticles d). different molar ratios of peptide to porphyrin in different samples e). fluorescence intensity changes in different samples f). comparison of singlet oxygen evolution . Adopted from ref. 118


2.6. Conclusions

Peptides serve as a versatile building block in designing minimalistic versions of complex biological systems. Programmable primary peptide sequences obtained from 20 gene coded amino acid can lead to the formation various nanoarchitectures. The different types of morphologies can be obtained by simply varying the amino acid sequence of the peptide. These different nanostructures can be generated from linear, cyclic or capped with synthetic aromatic moiety to enhance their assembly and to introduce functionality. They are potentially ideal structural and functional candidates for many applications such as biomaterials chemistry, food and cosmetic industry and catalysis. The self-assembly of short peptides consists of two or three amino acids is of particular interest due to their structural simplicity and availability.

Due to its ability to form a variety of different nanoarchitectures, peptides provide excellent supramolecular scaffolds to organize functional molecules such as porphyrin. The peptide mediated porphyrin nanostructures can facilitate the appropriate alignment of porphyrin molecules by flexible non-covalent connections. These soft interactions facilitates easy accessibility to the functional moiety. Peptide-porphyrin co-assembled nanoarchitectures have been utilized in many areas including light harvesting, photodynamic therapy and catalysis with improved performance.
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3. Fmoc-dipeptide/Porphyrin Molar Ratio Dictates the Efficiency of Energy Transfer
Nanostructures Produced by Biocatalytic Co-assembly

This work was submitted in part as: Nadeesha K. Wijerathne, Mohit Kumar and Rein. V. Ulijn

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N.K.W. and R.V.U. conceived, designed the experiments, analysed the data and wrote the paper.
N.K.W. prepared hyrogels performed the AFM, HPLC, FTIR, CD, UV-VIS, Fluorescence and
rheological Experiments, M.K. performed the TEM and confocal imaging analysis
3.1 Introduction

Self-assembly of \( \pi \)-conjugated chromophores is of interest as a minimalistic, biomimetic approach to produce optical and electronic nanostructures.\(^1\) An important source of inspiration is light harvesting in photosynthesis, which is achieved by precisely controlled assembly of light harvesting chlorophyll molecules templated by a protein matrix. In synthetic mimics, it has been challenging to achieve such precise and efficient arrangement of the light harvesting and energy transfer moieties, due to limited control over positioning of the chromophore molecules.\(^2\)\(^-\)\(^6\) Various self-assembled scaffolds have shown the potential to facilitate spatial control of chromophores towards efficient utilization of the broadband solar radiation, by means of energy transfer from a pre-excited donor to a ground state acceptor.\(^7\)\(^-\)\(^9\) The two critical factors for efficient Förster-resonance energy transfer (FRET) are the distance between donor-acceptor pairs which should be within 10 nm and good spectral overlap between the emission band of donor and the absorption band of acceptor.\(^10\) This can be achieved by non-covalent organization of appropriate donor-acceptor pairs in supramolecular assemblies.

Synthetic porphyrins are attractive chromophores for artificial photosynthetic systems due to their structural similarity with chlorophyll, availability of a wide range of derivatives, and well-understood photophysics and photostability.\(^11\)\(^-\)\(^15\) Porphyrins may either be covalently or non-covalently incorporated into self-assembling scaffolds to achieve the required organization.\(^16\)\(^-\)\(^25\) Non-covalent co-assembly of porphyrins within peptides is of particular interest due to diverse structural possibilities of peptides, which can lead to controlled organization of porphyrin molecules.\(^26\)\(^-\)\(^29\) In particular, very short peptides and peptide derivatives that are just 2-3 amino acids long are scalable and their assembly can be controlled by composition and sequence of amino
acids.\textsuperscript{30-38} They have previously been used to organize chromophores for the development of photo-responsive, light harvesting and electronically active materials.\textsuperscript{39-41}

A general challenge in the formation of optimized peptide/porphyrin nanostructures for efficient energy transfer is that they require controlled positioning of porphyrin within the supramolecular scaffold. In particular, when assembling hydrophobic (porphyrin) molecules in aqueous media, a cooperative assembly process can lead to the formation of kinetic aggregates, in addition to the desired thermodynamically favored structures. As a result, attempts to control their organization have been successful only in specific non-physiological conditions such as low pH (pH <2) and with organic co-solvents such as hexafluoroisopropanol (HFIP) to aid dissolution of hydrophobic components.\textsuperscript{22, 27} We have previously demonstrated the use of reversible enzymatic peptide bond formation coupled to in situ peptide self-assembly.\textsuperscript{42} This approach was shown to be fully reversible and consequently reduces the formation of kinetic aggregates, enabling formation of thermodynamically optimized functional energy transfer nanostructures, as previously demonstrated for supramolecular naphthalene di-imide/ peptide conjugates.\textsuperscript{35} We envisage that such a dynamic supramolecular scaffold can control the organization of porphyrin molecules while avoiding orthogonal assembly, directed by $\pi-\pi$ interactions between Fmoc moiety and porphyrins.\textsuperscript{43}
Here we demonstrate the use of reversible biocatalytic self-assembly to form Fmoc appended di-peptide/porphyrin co-assembled hydrogels in aqueous medium (in pH 8 phosphate buffer) where the organization of porphyrin within the peptide scaffolds can be modulated by varying the ratio of porphyrin and peptide precursor molecules.

### 3.2. Objectives

The three objectives of this work are

(i) To assess the use of reversible biocatalytic assembly to direct the co-assembly of porphyrins and Fmoc-peptide into supramolecular structures.

(ii) To systematically modulate the inter-porphyrin interactions within the peptide nanostructures by varying porphyrin/peptide ratios.
(iii) To investigate the light harvesting capability of the system by measuring energy transfer efficiency between Fmoc moiety and porphyrin molecules.

### 3.3 Results and Discussion

#### 3.3.1. Hydrogel Preparation and Characterization

We build on previous report from our group showing that Fmoc-T and L-NH₂ can be directly coupled by protease thermolysin to form Fmoc-TL-NH₂ nanofibers stabilized by aromatic-aromatic and hydrogen bonding interaction.⁴⁴ For the generation of peptide-porphyrin hydrogels, solutions of Fmoc-threonine (Fmoc-T, 20 mM) and Leucine amide (L-NH₂ 80 mM) and 1 mg thermolysin were mixed by vortexing for 10 seconds and sonicating for 1 minute in 1 mL of 100 mM sodium phosphate buffer (pH 8) solution at room temperature.⁴⁴ A transparent hydrogel was spontaneously formed within 30 minutes after mixing. Five different concentrations (0.01 mM, 0.1 mM, 0.2 mM, 1 mM and 2 mM) of 4,4’,4”,4”’-(Porphine-5,10,15,20-tetrayl)tetrakis(benzoic acid) (TCPP) were then introduced in this system (Scheme 3.1). In each case, a self-supporting hydrogel with a clearly visible gradual increase in coloration reflecting TCPP solution was generated and the gelation was completed in one hour (Figure 3.2. a. and b.). It was observed that

![Figure 3.1](image-url)
the gelation time increases in the presence of increasing concentrations of TCPP due to the incorporation of porphyrin molecules in to the peptide nanofibers.

The percentage conversion to Fmoc-TL-NH$_2$ was monitored over time using HPLC (**Figure 3.2. c. and b.**). Maximum conversions between 62-65% were observed after 96 hours (4 days) of reaction, which most likely represents the equilibrium conversion of the reversible amide condensation/hydrolysis reaction (**Figure 3.2. a.**).$^{42}$ It was observed that the varying concentration of TCPP had no systematic effect on the overall time dependent formation (in terms of reaction yield) of Fmoc-TL-NH$_2$/ TCPP with respect to concentration employed suggesting that peptide scaffold mainly remains unperturbed upon TCPP incorporation.

**Figure 3.2:** Time dependent HPLC demonstrating a). percent conversion b). chromatograms of Fmoc-TL-NH$_2$ formation over time
3.3.2. Microscopic Analysis

3.3.2.1 Atomic Force Microscopy (AFM)

Atomic Force Microscopic (AFM) analysis was performed in order to compare the morphologies of the TCPP/Fmoc-TL-NH$_2$ hydrogel at five different TCPP concentrations. The AFM studies showed the formation of a network of fiber bundles consisting of twisted fibers, which to our advantage did not significantly affect the hydrogel network of Fmoc-TL-NH$_2$ (Figure 3.3.), suggesting successful co-assembly.

![Figure 3.3](image)

**Figure 3.3:** Atomic Force Microscopic (AFM) images of nanofibers formed (i). 0 mM, (ii). 0.02 mM, (iii). 0.1 mM, (iv). 0.2 mM, (v). 1 mM, (vi). 2 mM (scale bar 250 nm). 100 fibers were measured to generate the histogram for each concentration.

The time dependent monitoring of AFM imaging was carried out to investigate the fiber formation pathway. It was observed that the initially formed spherical aggregates converts into fibers overtime forming a fiber network after 4 days (Figure 3.4.).

![Figure 3.4](image)

**Figure 3.4:** Time dependent AFM monitoring of a). spherical aggregates to b). fibers. Scale bar 250 nm
3.3.2.2. Transmission Electron Microscopy (TEM)

Transmission Electron Microscopic (TEM) images were acquired to further examine fibrous nanostructures and confirm the observations by AFM (Figure 3.5.). Twisted nanofibers were observed in both Fmoc-TL-NH₂ and Fmoc-TL-NH₂ / TCPP hydrogels.

![TEM images of hydrogels](image)

**Figure 3.5:** TEM images of a). 0 mM, b). 0.1 mM and 2 mM hydrogels. Inset shows the twisted nanofibers

Formation of TCPP co-assembled Fmoc-TL-NH₂ nanofibers was also confirmed by observing hydrogel samples under UV light with and without TCPP (at 345 nm) (Figure 3.1 a) and obtaining confocal microscopic images (Figure 3.3 b.).

3.3.2.3. Confocal Imaging

Porphyrin incorporated hydrogel sample showed red emission in the presence of UV radiation (Figure 3.6.) and the confocal image which was collected at the porphyrin region (405 nm) showed the presence of fibrous nanostructures, thus proving that the TCPP molecules are indeed incorporated into the Fmoc-TL-NH₂ nanofibers.
Fourier Transform Infrared (FTIR) spectroscopic analysis was carried out to investigate the propensity of hydrogen bonding between the amide-backbone of Fmoc-TL-NH₂ molecules. Strong absorbance of ~1625 cm⁻¹ and ~1680 cm⁻¹ in the amide I region demonstrated the hydrogen bond formation through amide and carbamate groups respectively. All supramolecular hydrogels, with and without the presence of TCPP at varying concentrations exhibited similar FTIR spectra (Figure 3.7.) suggesting that incorporation of TCPP does not have significant impact on the peptide/peptide backbone interactions, as discussed later. This observation indicates that the hydrogen bonding between the peptide backbones leading to an intrinsic β-sheet-like structure is maintained after co-assembly of porphyrin molecules.
Circular Dichroism (CD) spectroscopy was used to assess the helical organization of aromatic groups within the nanofibers both in the absence and presence of incorporated TCPP molecules. Self-assembled Fmoc-TL-NH₂ molecules showed non-zero signal in the amide (200-250 nm) and Fmoc regions (275-375 nm) of the spectrum, and the TCPP incorporated hydrogel also exhibited helicity in the porphyrin region (426 nm) (Figure 3.8.). When the CD signal intensities at 426 nm were compared for five concentrations of TCPP employed, it was observed that the helicity in the porphyrin region was enhanced with increasing concentration of TCPP suggesting that molecules

**Figure 3.7:** FTIR spectrum comparison of different concentrations of porphyrin with 20 mM peptide

3.3.4. Circular Dichroism (CD) spectroscopy
are incorporated into the chiral fiber. Thus, the chiral Fmoc-TL-NH₂ nanofibers induced helical

**Figure 3.8:** CD spectrum comparison of different concentrations of porphyrin with 20 mM peptide

**Figure 3.9:** CD signal intensity in the porphyrin absorbance wavelength at 426 nm showing two distinct regions
arrangement of achiral porphyrin molecules within the fiber network.\textsuperscript{46}

A plot of CD signal at 426 nm versus concentration of TCPP shows two distinct regions with a clear change in slope, with a transition point at 0.2 mM with 1:100 peptide to porphyrin molar ratio (Figure 3.9.).

### 3.3.5 Concentration Dictated Organization of Porphyrin

Based on both FTIR and CD observations, we believe that the differentiation in signal intensities are due to the degree of cooperative assembly versus self-aggregation of TCPP in the system, as shown schematically in Scheme 3.1.b. We propose that at lower concentrations, below the critical aggregation concentration of TCPP (0.2 mM), co-assembly is favorable, thus resulting in enhanced chiral orientation from Fmoc peptide to porphyrin. At higher concentrations, self-aggregation of achiral TCPP is dominant, showing reduced helicity induction in the presence of more TCPP. The model is in agreement with the FTIR observations: in the lower concentration range where peptide: porphyrin molar ratio ranges from 1000:1 to 100:1, the percentage of porphyrin molecules does not have a significant effect on hydrogen bonding of the peptide backbone as explained by FTIR analysis. At higher concentrations of porphyrin, TCPP molecules separately self-aggregate, therefore there is no interference with the β-sheet structure. We propose that the TCPP molecules are most likely incorporated via π-π interactions involving fluorenyl groups of Fmoc moiety and aromatic regions of porphyrin molecules while leaving the β-sheet-like arrangement of the Fmoc-TL-NH$_2$ assemblies intact (Scheme 3.1.b).
3.3.5.1. Fluorescence Spectroscopy

In order to investigate the interaction between TCPP monomers and Fmoc-TL-NH₂ supramolecular assemblies and to further probe the two-stage assembly, fluorescence spectroscopic analysis was carried out. Time-dependent fluorescence monitoring during the biocatalytic formation of the gels for Fmoc-TL-NH₂ and TCPP molecules illustrated extended π-π interactions over time as evident from the changes in the signal intensity. Upon excitation at 287 nm, Fmoc monomers exhibit a strong emission at 320 nm, which started to decrease, and red shift as the self-assembly progresses. Additionally, a sharp reduction in shoulder peak at ~ 370 nm which corresponds to the decrease in spherical aggregates formed by Fmoc-T precursors was observed and the appearance of new peak at ~ 450 nm indicated the formation of extended π-π stacking interactions in Fmoc-TL-NH₂ nanofibers.

Figure 3.10: Time dependent fluorescence monitoring of extended π-π stacking interactions formation a). without porphyrin b). with porphyrin collected at 287 nm with 20 mM Fmoc-TL-NH₂ and 2 mM TCPP
The changes observed in fluorescence emission were comparable in hydrogels in the presence (Figure 3.10.b) and absence of porphyrin (Figure 3.10.a). The transition from spherical aggregates to fibers was also confirmed by time dependent AFM images (Figure 3.4.).

In order to investigate the degree of π-π interaction between Fmoc groups after incorporation of TCPP into the fiber network, the fluorescence intensities at 450 nm were compared with respect to the TCPP concentration (Figure 3.11.a and Figure 3.12.a.). A decrease in fluorescence intensity with increasing TCPP concentration suggested that the extent of Fmoc π stack is directly affected by the amount of TCPP molecules being incorporated into the nanofibers. This observation further confirms that the co-assembly of TCPP molecules into Fmoc-TL-NH₂ nanofibers occurs via the insertion of porphyrin monomers into the Fmoc π- stack. Furthermore, it was also observed that the porphyrin emission at 655 nm is enhanced with increasing concentration of TCPP (Figure 3.11.b. and Figure 3.12.b.).

**Figure 3.11:** Fluorescence spectra a). for Fmoc region collected at 287 nm b). for TCPP region collected at 417 nm
The plots of emissions at 450 nm and 650 nm against concentration of TCPP also show two distinguishable regions, corresponding to co-assembly of monomeric TCPP molecules into the fiber network at lower concentrations and self-aggregated monomers at higher concentrations of TCPP (Figure 3.12.a. and b.), similar to the observation in CD (Figure 3.9.). These data suggest that organization of TCPP into peptide fiber network can be significantly controlled by their concentration, where lower concentrations show more monomeric incorporation of TCPP as a co-

![Graph A] ![Graph B]

Figure 3.12: Fluorescence signal intensity a). at 450 nm describing the extent formation of extended π- π stacking interaction b). at 655 nm corresponding to the increase in signal intensity in porphyrin region

assembly and higher concentration demonstrate self-aggregation of TCPP. The critical concentration of TCPP to initiate the self-aggregation found to be beyond 0.2 mM as observed by the variations in the emission intensity at 655 nm (Figure 3.12.b.).

3.3.5.2. Rheology Analysis

The mechanical properties of bulk gel samples were assessed by rheology. It was demonstrated that the strength of the gel decreased with the increasing concentration of porphyrin in co-assembled peptide-porphyrin nanostructures at the five different concentrations of TCPP (Figure
3.13.a). We propose that the presence of self-aggregated porphyrins at the higher concentration of TCPP weakens the interaction between fibers inside the hydrogel compared to that of co-assembled nanofibers. The network properties of the gel turn out to be directly affected by the amount of porphyrin molecules bound into the supramolecular nanofibers (Figure 3.13.b.). The rheological data propose that the co-assembly and self-aggregation of porphyrin inside the hydrogel have an effect on network arrangement of fibers in space, including the strength and amount of crosslinking of fibers.

![Image](image_url)

**Figure 3.13:** a). Storage modulus (G’) and Loss modulus (G’’) of Fmoc-TL-NH₂ (20 mM) and Fmoc-TL-NH₂/TCPP with five different concentrations. 0 mM represents the Fmoc-TL-NH₂ hydrogel without TCPP b). Storage modulus (G’) comparison at different concentrations of TCPP co-assembled into Fmoc-TL-NH₂ fibers.

3.3.6. **Energy transfer Analysis and Efficiency Calculations**

3.3.6.1. **Absorbance Spectroscopy**

Absorbance and fluorescence were further investigated to study the energy transfer capability of the hydrogel. Incorporation of TCPP molecules into fiber network was confirmed by a shift in the
absorption spectrum of TCPP upon the formation of Fmoc-TL-NH₂/TCPP nanofibers. The shifts in the absorbance of Soret-band (406 nm) with respect to the TCPP monomers were investigated with the five different concentrations (Figure 3.14 a.). It was noted that the red-shift in the absorption maximum at 406 nm was inversely correlated to TCPP concentration employed, indicating that higher concentrations give rise to less effective energy transfer (Figure 3.15.). In order to confirm the proposed concentration-dependent co-assembly, temperature dependent absorbance spectroscopic study was carried out. A red-shift in absorbance of the Soret-band with increasing temperature for 0.1mM TCPP confirms that red-shifts are obtained upon dis-assembly of TCPP aggregates (Figure 3.14.b.).

**Figure 3.14:** Absorbance spectra of a). Fmoc-TL-NH₂ (20 mM) and Fmoc-TL-NH₂/TCPP with five different concentrations for TCPP region. 0 mM represents the Fmoc-TL-NH₂ hydrogel without TCPP b). temperature dependent TCPP with 0.1 mM TCPP solution in pH 8 0.1 M phosphate buffer.
Fluorescence spectroscopy was further studied to investigate the energy transfer between Fmoc moiety and TCPP monomers at varying concentration ratios. When the sample was excited at the Fmoc region (287 nm), an emission around 655 nm was detected, corresponding to the emission from TCPP molecules (Figure 3.11.a and Figure 3.18.a.), due to efficient energy transfer from Fmoc groups of Fmoc-TL-NH2 molecules to incorporated TCPP molecules. A good overlap between the emission spectrum of Fmoc (295 nm – 500 nm) and absorption spectrum of TCPP (400 nm - 450 nm) can facilitate the Förster-resonance energy transfer (FRET) between Fmoc (donor) and TCPP (acceptor) (Figure 3.16.a.). Control measurements were done using the hydrogel without TCPP and a solution containing free TCPP molecules (Figure 3.18.a. and b.). Absence of signal corresponding to TCPP at 655 nm when the Fmoc molecules were excited at 287 nm, confirmed that the energy transfer takes place only inside peptide-porphyrin supramolecular assemblies.

Figure 3.15: Red shift in the soret-band of TCPP in the hydrogels compared to TCPP monomers (409 nm)

3.3.6.2. Energy Transfer Study by Fluorescence spectroscopy

Fluorescence spectroscopy was further studied to investigate the energy transfer between Fmoc moiety and TCPP monomers at varying concentration ratios. When the sample was excited at the Fmoc region (287 nm), an emission around 655 nm was detected, corresponding to the emission from TCPP molecules (Figure 3.11.a and Figure 3.18.a.), due to efficient energy transfer from Fmoc groups of Fmoc-TL-NH2 molecules to incorporated TCPP molecules. A good overlap between the emission spectrum of Fmoc (295 nm – 500 nm) and absorption spectrum of TCPP (400 nm - 450 nm) can facilitate the Förster-resonance energy transfer (FRET) between Fmoc (donor) and TCPP (acceptor) (Figure 3.16.a.). Control measurements were done using the hydrogel without TCPP and a solution containing free TCPP molecules (Figure 3.18.a. and b.). Absence of signal corresponding to TCPP at 655 nm when the Fmoc molecules were excited at 287 nm, confirmed that the energy transfer takes place only inside peptide-porphyrin supramolecular assemblies.
3.3.6.3. Percent Energy Transfer Calculation

A further energy transfer study was carried out to calculate the percent efficiency of energy transfer between Fmoc moiety and TCPP molecules at different ratios. The excitation spectra were collected for the five different concentrations of TCPP by measuring the emission of the samples at 655 nm (porphyrin region) and was compared with the absorbance spectra of respective hydrogel

![Absorbance and Intensity Spectra](image.png)

**Figure 3.16:** a). Spectral overlap between Fmoc emission and porphyrin acceptor b). Florescence spectra of Fmoc-TL-NH₂ (20 mM) and Fmoc-TL-NH₂/TCPP with five different concentrations for TCPP region collected at 287 nm showing the porphyrin emission by energy transfer. 0 mM represents the Fmoc-TL-NH₂ hydrogel without TCPP samples (**Figure 3.17.**).

It was observed that there exists a deviation from linearity with respect to the TCPP concentration used for co-assembly with a higher slope at lower concentrations in energy transfer efficiency (**Figure 3.18.c.**). The hydrogel sample containing with 10:1 (peptide: porphyrin) molar ratio exhibited a 40% showing the highest energy transfer efficiency (**Figure 3.18.c.**). Interestingly, at the transition point where peptide to porphyrin ratio is 1:100 with 0.2 mM TCPP, nearly 10% efficiency in energy transfer was detected, despite the low number of porphyrin molecules present.
in the system. This is due to the well-ordered TCPP molecules inside the peptide nanofibers proving the close proximity of TCPP molecules arranged by the Fmoc-TL-NH$_2$ molecules. To facilitate the FRET the donor and acceptor molecules should be 1 to 10 nm apart and this distance between chromophore molecules provides the perfect organization for light harvesting.$^{10}$ The advantage of this type of energy transfer is its ability to utilize a broad range of light; both in ultraviolet (UV) and visible regions of the electromagnetic spectrum, to excite TCPP molecules for enhanced light harvesting.

**Figure 3.17:** FRET efficiency calculation. Comparison between Fmoc emission and excitation spectra of 20 mM Fmoc-TL-NH$_2$ and a). 2 mM b). 1 mM c). 0.2 mM d.) 0.1 mM and e). 0.02 mM TCPP
In conclusion, we have demonstrated the reversible biocatalytic co-assembly of Fmoc-dipeptide/porphyrin system, which gives rise to nanofibrous hydrogel with energy transfer capability. Ordered nanofibers were formed after incorporation of porphyrin molecules into peptide nanofibers stabilized by H-bonding and π-stacking interactions of Fmoc moieties. It was observed that the co-assembled porphyrin molecules are arranged chirally within the peptide nanofibers. We detected two distinct regions corresponding to co-assembly and self-aggregation porphyrin molecules depending on the concentration employed in incorporation, hence providing a control over the organization of porphyrin molecules in the di-peptide supramolecular assemblies. There is an efficient energy transfer, taking place between the Fmoc moiety and porphyrin molecules when probed via fluorescence emission and excitation measurements. In spite of low peptide to porphyrin molar ratio, the co-assembling region exhibited substantial energy transfer.

Figure 3.18: Energy transfer between Fmoc moiety and TCPP molecules in 20 mM Fmoc-TL-NH$_2$ / 2 mM TCPP nanofibers, Emission spectra a). collected at 287 nm, b). inset of TCPP region collected at 287 nm and c). percent FRET efficiencies with respect to the TCPP concentration in nanofibers

3.4. Conclusion
transfer efficiency due to the organization porphyrin molecules by peptide scaffolds and these hydrogels will provide a UV sensitive light harvesting material in the future.

3.5. Experimental

3.5.1. Materials

Fmoc-T, TCPP (porphyrin derivative), Acetonitrile and trifluoroacetic acid was purchased from Sigma-Aldrich (USA). L-NH₂ was purchased from Fisher Scientific (USA). Thermolysin (from bacillus Thermoproteolyticus rokko) was obtained from Nova Biochem (batch 079K1706 mol wt 34.6 kDa by amino acid sequence). All the chemicals and solvents were used as purchased unless otherwise stated.

3.5.2. Methods

3.5.2.1. Preparation of Fmoc-TL-NH₂ and Fmoc-TL-NH₂/porphyrin hydrogels

For the generation of Fmoc-TL-NH₂ hydrogel, Fmoc-T and L-NH₂, were mixed in a ratio of 20:80 mM with in a glass vial. 1 mL of 0.1 M sodium phosphate buffer of pH 8 was added to the mixture with 1 mg of thermolysin, vortex mixed for 10s and sonicated for 1 min. Samples were then let stand for 4 days in room temperature to allow the full self-assembly conversion. For the formation of Fmoc-TL-NH₂/porphyrin hydrogels, Fmoc-T, L-NH₂ and TCPP were mixed in a ratio of 20:80:2 mM with in a glass vial and followed the same procedure as for Fmoc-TL-NH₂ hydrogel.

3.5.2.2. HPLC

50 μL aliquots of each sample were mixed with 950 μL of 50:50 acetonitrile:water (Sigma-Aldrich) solution containing 0.1% trifluoroacetic acid (Sigma-Aldrich). An aliquot of 50μL was injected into a Dionex P680 system operating with a Macherey-Nagel 250 A#, 4.6 250 mm, C18
column was used for reversed phase HPLC. A water and acetonitrile mobile phase was used ramped from 20-80% for 20 minutes with a flow rate of 1mL min\(^{-1}\). The detector was UVD170U UV-Vis detector and Fmoc-peptide amphiphiles and porphyrin were at a 287 nm and 417 nm wavelengths respectively.

### 3.5.2.3. UV-VIS spectroscopy

1ml samples prepared in 0.1 quartz cuvette were used measure the UV-VIS absorbance spectra with a Jasco V-660 spectrophotometer at a scanning speed of 400 nm min\(^{-1}\). Measurements were collected between 350-800 nm wavelengths.

### 3.5.2.4. Fluorescence spectroscopy

1ml samples prepared in 10 mm Styrofoam cuvette were used measure the fluorescence emission spectra with a Jasco FP-8500 spectrofluorometer at a scanning speed of 500 nm min\(^{-1}\). Fmoc- TL-NH\(_2\) samples were excited at 287 nm and were recorded between 290-600 nm using a bandwidth of 5 nm with a medium response and 1 nm data pitch. For porphyrin excitation wavelengths was 417nm with a recoding wavelength of 600-800nm with same bandwidth, response and data pitch values.

### 3.5.2.5. AFM

Images were acquired using a Bruker Dimension AFM system. AFM samples were prepared using 20 \(\mu\)L of 20mM Fmoc- TL-NH\(_2\)/TCPP hydrogel solution on freshly cleaved mica. Analysis was done on dried samples on mica substrates and imaged in scan-assist air mode.

### 3.5.2.6. Circular Dichroism
1mL samples prepared in 0.01 quartz cuvette were used measure the CD signal with a Jasco V-660 spectrophotometer at a scanning speed of 400 nm min\(^{-1}\). Measurements were collected between 190-800 nm wavelengths

**3.5.2.7. Confocal fluorescence imaging**

Confocal imaging was performed using Zeiss LSM 880 AIRYSCAN FAST LIVE CELL instrument. 405 nm laser was used as excitation source. The images were collected from 600 nm-700 nm.

**3.5.2.8. Rheology Measurements**

Rheological properties were measured with an Anton Paar MCR 302 rheometer with temperature controlled at 25 \(^\circ\)C using a 10 mm vane geometry. First amplitude sweeps were performed at a constant frequency of 1Hz from sheer strain 0.01-100% to ensure the measurements to be taken in viscoelastic regime. The frequency sweep was done to measure G\(^{'}\) and G\(^{''}\) at constant strain value in the frequency range of 0.1-100 Hz. All the samples were 4 days old and transferred on to the stage before the measurements.

**3.5.2.9. Transmission electron microscopy**

Carbon-coated grids were purchased from Electron Microscopy Sciences. A drop (5 µl) of the sample solution was applied to the carbon-coated grid and incubated for one minute. Excess solution was removed by blotting the grid with a piece of filter paper, followed by staining with 5 µl of 2\% (w/v) uranyl acetate solution for 30 seconds. After blotting excess stain solution, the grid was left to air dry. The negatively stained sample was imaged in FEI TITAN Halo TEM operating
at 300 kV. Images were recorded in the low-dose mode (20 e− Å−2) on an FEI CETA 16M camera (4,096 × 4,096 pixels).
3.6. References


4. Customizable Nanostructures by Spontaneous Aminolytic Cyclization and Self-Assembly of Dipeptide Methylesters in Water

This work will be submitted in part as: Nadeesha K. Wijerathne, Charalampos G. Pappas, Jugal Kishore Sahoo, Ankit Jain, Daniela Kroiss, Ivan R. Sasselli, Ana Pina, Ayala Lampel and Rein V. Ulijn, *ACS Applied Material Interfaces*

N.K.W. C.G.P. and R.V.U. conceived, designed the experiments, analyzed the data and wrote the paper. N.K.W, prepared hyrogels performed the HPLC, LC-MS, FTIR, CD, UV-VIS, AFM, TEM and rheological Experiments C.G.P. initial HPLC analysis, J.K.S., help wrote the paper, A.J. synthesized Fe$^{III}$-TMPyP and performed TEM imaging, D.K. performed Mass Spectroscopy, I.R.S., A.P., and A.L., analyzed the data
**4.1. Introduction**

Peptides and peptide derivatives are attractive building blocks for the fabrication of artificial nanostructures with tremendous biological and nanotechnology applications, arising from their combinatorial diversity and biocompatibility.\(^1\)\(^{-8}\) Peptide sequences as short as two or three amino acids have been utilized for nanostructure formation in a sequence dependent manner using linear\(^9\)\(^{-14}\) or cyclic peptides.\(^15\)\(^{-18}\) Cyclic dipeptides (or diketopiperazines) involve the presentation of amino acid side chain functionality at the exterior of the nanostructure, thus rendering it accessible for interactions and/or functionalization. In particular Govindaraju’s group has extensively investigated their assembly in organic media, highlighting interesting properties of these systems, including the increased stability towards proteolysis.\(^19\)\(^{-21}\) Both supramolecular organogels\(^22\) and hydrogels\(^23\) have been reported based on these structures. Gazit and Reches demonstrated the spontaneous formation and self-assembly of surface-bound arrays of cyclic diphenylalanine peptide nanotubes using chemical vapor deposition.\(^24\) More generally, cyclic dipeptides (especially aspartame) are well-known byproducts resulting from chemical degradation, through aminolysis of dipeptide esters, suggesting that they form spontaneously under much milder, aqueous conditions.\(^25\)\(^{-26}\)
Scheme 4.1: (A) Spontaneous aminolysis of dipeptide methyl esters to form cyclic self-assembling moieties through intermolecular cyclisation in aqueous phosphate buffer (100 mM sodium phosphate buffer pH 8) at room temperature. Chemical structures of the dipeptide methyl ester and cyclic building blocks with different amino acid side chains depicted by single letter code, with their self-assembly propensity to form supramolecular stacks through hydrogen-bonding. (B) Schematic representation of the supramolecular organization of different cyclic dipeptides showing sequence-specific morphologies. D-amino acids (for c[LF]) are represented with open circles.
We decided to investigate whether this known autonomous reaction\textsuperscript{25} can be optimized and coupled with the use of dipeptide sequences that give rise to spontaneous nanostructure formation in water, without the need of complex chemical synthesis\textsuperscript{23} or other physicochemical routes not always compatible with self-assembly.\textsuperscript{24} This approach combines the advantages of \textit{in situ}, chemically activated assembly (\textbf{Scheme 4.1. a.}) and the versatility of supramolecular nanostructures based on cyclic dipeptides to produce functional minimalistic supramolecular systems (\textbf{Scheme 4.1. b.}). Furthermore, we have shown that spontaneous assembly in water, combined with their amphiphilicity may be exploited to form co-assemblies with functional molecules like porphyrins, leading to the formation of catalytic nanofibers with enhanced peroxidase activity.

\textbf{4.2. Objectives}

We have following objectives set to study

\begin{enumerate}
  \item \textit{In situ} cyclization of linear di-peptide methyl esters in aqueous medium and propensity of spontaneous self-assembly of cyclic di-peptides depending on their sequence and their morphological differences.
  \item The peptide-mediated co-assembly of metalloporphyrin molecules into the peptide nanostructures.
  \item Peroxidase activity of peptide-porphyrin co-assembled nanostructures in oxidizing small organic molecules.
\end{enumerate}

Cyclic supramolecular structures are resistant to proteolysis from enzymes, and they lack terminal ionic groups, which suggest that they exhibit enhanced stability and versatility compared to linear sequences. The straightforward and versatile approach reported here to
generate these self- and co-assembling functional nanostructures might pave the way for using them in a variety of applications in biomedicine and nanotechnology.

4.3. Results and Discussion

4.3.1. Spontaneous Cyclization of Linear Di-Peptide Methylesters and In-situ Self-assembly of Cyclic di-peptides

4.3.1.1. HPLC and Mass Spectroscopic Analysis

We started with an amphiphilic sequence, the well-known dipeptide ester, aspartame-DF-OMe. While we observed no noticeable macroscopic differences over time when incubating aspartame in aqueous phosphate buffer, analysis by HPLC and mass spectrometry revealed the formation of the cyclic dipeptide (c[DF]) to near-complete conversion within 24 hours (Figure 4.2.). Building on this observation, we then investigated a variety of homo- and hetero dipeptide methyl esters (R₁R₂-OMe), varying the amino acid side chain (R₁,₂ = L, F) those having different aggregation potentials. Macroscopic changes could be observed with LF-OMe, DLPF-

![Figure 3.1: a). Chemical structure of different cyclic dipeptides b). digital micrographs of various cyclic dipeptides showing sequence specific macroscopic behavior (Images captured 72h after reaction).](image-url)
OMe, LL-OMe and FF-OMe showing the formation of gels, crystalline solution and precipitate respectively. (Figure 4.1.b.) which turned out to be a consequence of the formation of supramolecular assemblies of varying morphologies (fibres, tapes and tubes), as confirmed by Atomic Force Microscopy (AFM) and Transmission Electron Microscopy (TEM).

![Figure 4.1.b.](image)

**Figure 4.1.b:** Time-dependent HPLC (monitored at 225 nm) a). percent conversion and b). HPLC chromatograms of formation of cyclic dipeptides from corresponding dipeptide methyl esters in 100 mM sodium phosphate buffer at room temperature (pH 8)

In all cases, the cyclic dipeptides formed in high yield (as confirmed by high resolution MS (Figure 4.4.) and LC-MS (Figure 4.3.)). The concentration used for all the dipeptide methyl esters was 20 mmol kg\(^{-1}\), with all the reactions performed in 100 mM sodium phosphate buffer pH 8.0 at room temperature. Samples were vortexed for 60s and sonicated for 120s in order to obtain a homogenous solution and left standing at room temperature. For hydrophobic dipeptides, dramatic changes were macroscopically observed. In the case of LF-OMe, a translucent gel-like material was formed after 24 hours, while from homo-dipeptide esters
(c[LL] and c[FF]), precipitate-like structures were noticed (Figure 4.1). As expected, stereochemistry does not play a role here and

Figure 4.3: LC-MS spectra for cyclic dipeptides, LC chromatograms (Top) MS spectrum (Bottom)
Figure 4.4: High resolution mass spectra of a). c[DF], b). c[LF], c). c[DLDF], d). c[LPF], d). c[LL] and f). c[FF]
for the dipeptide methyl ester containing D-amino acids \( ^{\text{D}}\text{L}^{\text{D}}\text{F-OMe} \), as a translucent hydrogel was noticed which could by visual inspection not be distinguished from that produced from the all \(^{\text{L}}\)-peptide (Figure 4.1). It is worth mentioning that formation of higher linear or cyclic oligomers under the conditions tested was not observed using HPLC/MS suggesting that only the cyclic di-peptide of interest will be formed.

4.3.2. Characterization of Cyclic Di-peptide Nanostructures

4.3.2.1 Fourier transform infrared spectroscopy (FTIR) Analysis

We investigated changes in amide conformation and interactions of the peptide backbones, as a result of spontaneous intermolecular dipeptide cyclisation using FT-IR spectroscopy (Figure 4.5).

**Figure 4.5:** FTIR spectra of the cyclic dipeptides a). at 10 minutes and after 72 hours of the reaction and b). comparison of cyclic dipeptides after 72 hours. Concentration used was 20 mmol kg\(^{-1}\) in phosphate buffer pH 8.

f). The FT-IR spectra of the dipeptide esters immediately after dissolving did not show
significant evidence of an ordered hydrogen bonding network (Figure 4.5 a-e.), while It is worth mentioning that the 1675 cm$^{-1}$ peak observed in c[FF] and c[LF] may be attributed to the presence of residual trifluoroacetic acid (TFA).$^9$ After the reaction was completed, two characteristic vibrations were observed for c[DF]. A peak at 1580 cm$^{-1}$, which can be assigned to carbonyl stretch of the carboxylate group, suggested deprotonation of the side chain and a second broad peak at 1650 cm$^{-1}$ revealing hydrogen-bonding interactions, involving the amide carbonyl groups. A broader peak with higher intensity was identified for both hydrogels c[LF] and c[PLpF], suggesting that aggregation takes place via intermolecular hydrogen bonding of the amide groups, giving rise to the formation of hydrogels.$^9$ For c[FF] and c[LL], hydrogen bonding interactions also played a role on the formation supramolecular structures.

4.3.2.2. Circular Dichroism (CD) Analysis

In order to gain insights into the supramolecular chirality of the cyclic dipeptides and the role of the chiral ordering of the amino acid residues on the spontaneous assembly, Circular Dichroism (CD) spectroscopy was also used (Figure 4.6. e). The CD spectrum of c[DF] showed a weak negative peak around 220 nm. A dramatic change was observed for c[LL], exhibited an intense negative CD signal, as this was evidenced by the appearance of a peak at 222 nm (Figure 1D), highlighting the supramolecular chiral ordering of leucine amino acid residues within the assembly. Reduced CD signals were observed for the c[LF] and c[PLpF] hydrogels, with opposite chirality.$^{27}$ The cyclic diphenylalanine (c[FF]) showed a positive peak around 220 nm. The CD spectra of the dipeptide esters immediately after dissolving showed limited chiral supramolecular arrangement (Figure 4.6. a-e.).
Time dependent AFM imaging was used to investigate the structural transitions at the microscopic level before and after the formation of the cyclic dipeptides (Figure 4.7). Analysis by AFM showed the formation of amorphous aggregates in case of c[DF], with no distinct supramolecular transition observed after 72 hours. In case of c[LF] and c[PLDF]), the dipeptide esters showed ill-defined aggregates. Over time, fibrillar structures were formed, which increased both in length and density during the formation of hydrogels. For the cyclic homodipeptides di-leucine (c[LL]) or di-phenylalanine (c[FF]), at the early of the process, sheet-like structures were detected. A supramolecular reconfiguration to form tape and tubular assemblies was noticed respectively, which is in agreement for the latter with previous observations using CVD.²⁴

Figure 4.6: CD spectra of the cyclic dipeptides a). at 10 minutes and after 72 hours of the reaction and b). comparison of cyclic dipeptides after 72 hours. Concentration used was 20 mmol kg⁻¹ in phosphate buffer pH 8.

4.3.2.3. Atomic Force Microscopy (AFM) Analysis

Time dependent AFM imaging was used to investigate the structural transitions at the microscopic level before and after the formation of the cyclic dipeptides (Figure 4.7). Analysis by AFM showed the formation of amorphous aggregates in case of c[DF], with no distinct supramolecular transition observed after 72 hours. In case of c[LF] and c[PLDF]), the dipeptide esters showed ill-defined aggregates. Over time, fibrillar structures were formed, which increased both in length and density during the formation of hydrogels. For the cyclic homodipeptides di-leucine (c[LL]) or di-phenylalanine (c[FF]), at the early of the process, sheet-like structures were detected. A supramolecular reconfiguration to form tape and tubular assemblies was noticed respectively, which is in agreement for the latter with previous observations using CVD.²⁴
Figure 4.7: Time-dependent AFM images of the cyclisation reaction after 10 minutes, 6 and 72 hours for c[DF], c[LF], c[DLDF], c[LL] and c[FF]
4.3.2.4. Transmission Electron Microscopy (TEM) Analysis

TEM images (Figure 4.8.) of the final assemblies further support the supramolecular structures found using AFM. The spectroscopic and microscopic findings highlight that the methyl ester precursors do not adopt well-defined supramolecular arrangements, however they contain sufficient chemical information to trigger the formation of diverse supramolecular structures (fibers, tapes and tubes), as observed for (non)-symmetrically hydrophobic dipeptides (c[LL], c[LF], c[DLDF], c[FF]) after cyclisation. For the negatively charged containing sequence (c[DF]) a less ordered supramolecular arrangement was observed.

4.3.2.5. Rheology Analysis

Having demonstrated the formation of supramolecular nanostructures by in situ cyclisation and assembly, we subsequently investigate the possibility to form hydrogels with tunable mechanical properties. We hypothesized that this should be feasible by simply varying the starting material concentrations. In order to demonstrate this, we monitored the stiffness of c[LF] hydrogels at four different concentrations (5, 10, 20 and 40 mmol kg\(^{-1}\)) using rheology. The photographic images and AFM images were also obtained for comparison (Figure 4.9). The AFM images
revealed that the formation of more dense fibers with increasing concentration (Figure 4.9.b). The 40 mmol kg\(^{-1}\) hydrogel exhibited almost three-fold increase in stiffness (G’ (storage modulus) ≈ 7000 Pa) compared to the 20 mmol kg\(^{-1}\) (G’ ≈ 2000 Pa). The 10 mmol kg\(^{-1}\) hydrogel showed a G’ of 1000 Pa, while the sample at the lowest concentration was not sufficiently stable for accurate analysis by rheology (Figure 4.10.a). These results may be of interests for the formation of cell

Figure 4.9: concentration dependent hydrogel formation of c[LF] a) photographic, b). AFM images. Scale bar 250 nm

Figure 4.10: a). Plot comparing the stiffness of different concentrations (10, 20 and 40 mmol kg\(^{-1}\)) of c[LF] gels b). Temperature sweep measurements and c). Self-healing measurements of 20 mmol kg\(^{-1}\) c[LF] in 100 mM sodium phosphate buffer pH 8 after 72 hours of the reaction.
culture matrices with tunable mechanical properties that could be used as scaffolds for stem cells differentiation. The 20 mmol kg$^{-1}$ hydrogel was investigated further to assess its thermal stability (temperature range from 25°C to 70°C) and self-healing propensity under a constant strain (0.3%). It was observed that the hydrogel retained its stability even at 70°C maintaining the stiffness (G’) unchanged (Figure 4.10. b.).

The self-healing ability was then performed by measuring G’ and G” at a constant stress for 120 seconds, six cycles at 120s intervals for recovery. The gel state and G’ were kept intact after completion of six cycles but the recovery time was increased in each cycle (Figure 4.10. c.).
4.3.3. Cyclic Di-peptide mediated Metalloporphyrin Nanostructures with Enhanced Peroxidase-Like Activity

Scheme 4.2: Schematic representation of the co-assembly propensity of dipeptide hydrogel c[LF] and metalloporphyrin (Fe$^{III}$–TMPyP) and the catalytic activity of c[LF]-Fe$^{III}$–TMPyP nanostructures on oxidation of pyrogallol.
4.3.3.1. Formation of Peptide-Porphyrin Co-assembled Nanostructures  Multi-component co-assembly has been used to generate new materials with enhanced complexity and functionalities.\textsuperscript{31-34} Consequently, we investigated whether spontaneous co-assembly may be used to introduce functionality into these structures. Thus, dipeptide methyl esters were used to investigate co-assembly with a cationic metalloporphyrin derivative,

![Figure 4.11](image_url)  

**Figure 4.11:** a) FT-IR spectra of c[LF] and b) AFM images generated from co-assembly between Fe\textsuperscript{III}-TMPyP (2 mM) and a. c[LF], b. c[PLPF] (20 mmol kg\textsuperscript{-1}) and c. Fe\textsuperscript{III}-TMPyP (2 mM). The digital images and spectra was taken after 72 hours of reaction. Scale bar 500 nm.
tetramethylpyridylporphyrin iron complex (Fe$^{III}$-TMPyP) (Scheme 4.2.), which has been previously reported to exhibit peroxidase-like activity when bound to an antibody.\(^{35}\) Out of six cyclic peptides in the study, c[LF] showed promising co-assembly behavior with Fe$^{III}$-TMPyP, without significantly disturbing the hydrogen-bonding pattern as evidenced using FT-IR spectroscopy (Figure 4.11. a.). Structural characterization using AFM (Figure 4.11. b-c) revealed that incorporation of Fe$^{III}$-TMPyP into c[LF] and c[PLDF], exhibited a fibrillar network with an apparently more aligned pattern (Figure 4.11. a-c.) compared to the fibers without porphyrin.

4.2.3.2. Investigation of peroxidase activity of Fe$^{III}$-TMPyP- c[LF] nanostructures

We then study the catalytic efficiency of peptide-metalloporphyrin complexes on the oxidation of pyrogallol (120 seconds) catalyzed by c[LF]-[Fe$^{III}$-TMPyP] nanostructures and c[LF] hydrogels containing Fe$^{III}$-TMPyP to spontaneous oxidation of pyrogallol. scale bar 500 nm.

**Figure 4.12:** a). AFM images of different concentrations of c[LF] i. 0 (control) ii. 1 iii. 2 and iv. 5 mmol kg$^{-1}$ co-assembled with 1 μM Fe$^{III}$-TMPyP, b). Time dependent oxidation of pyrogallol (120 seconds) catalyzed by c[LF]-[Fe$^{III}$-TMPyP] nanostructures and c). Comparison of initial rates of oxidation of pyrogallol using c[LF] hydrogels containing Fe$^{III}$-TMPyP to spontaneous oxidation of pyrogallol. scale bar 500 nm.
pyrogallol. The c[LF]-porphyrin nanofibers showed enhanced catalytic activity compared to Fe$^{III}$-TMPyP monomers, suggesting that the fibrous hydrogel network aids in accelerating the oxidation reaction than the Fe$^{III}$-TMPyP molecules present in solution (Figure 4.12.). We employed three different concentrations of c[LF] (1, 2 and 5 mmol kg$^{-1}$) in the presence of 1 μM solution of Fe$^{III}$-TMPyP. The peroxidase activity of resulting samples was tested for oxidation of pyrogallol by monitoring the oxidation product by time course UV-Visible absorbance at 420 nm (Figure 4.12 b.). The control measurements were carried out with a sample containing only Fe$^{III}$–TMPyP and another one with only substrate (1 mM pyrogallol) to ensure that the spontaneous oxidation of pyrogallol is not contributing to catalytic effects.

4.3.3.2. Calculation of Kinetic Constants for oxidation of pyrogallol

The hydrogel formed at 5mM c[LF], showed the highest initial rate of oxidation (Figure 4.12 c.). The variation of the initial rates may be attributed to the morphological changes as demonstrated using AFM, where increasing peptide concentration leads to the formation of more ordered assemblies, thus the well-aligned arrangement of Fe$^{III}$-TMPyP / c[LF] via nanofiber network enhanced its catalytic activity by providing access to more active sites for catalysis (Figure 4.12 a.). The rate of oxidation of pyrogallol was the lowest compared to the rates of porphyrin containing samples.

Furthermore, the Lineweaver-Burk plot was constructed for the 5 mmol kg$^{-1}$ c[LF] hydrogel and 1 μM Fe$^{III}$-TMPyP in order to calculate the steady-state kinetic parameters (Figure 4.13 a.). The Michaelis constant K$_m$ and the catalytic constant k$_{cat}$ values were found to be 3.4 mM and 158 min$^{-1}$ respectively. The efficiency of the process k$_{cat}$/K$_m$ (4.7 x 104 M$^{-1}$min$^{-1}$), is comparable to previous reports involving much more complex antibody stabilized porphyrin structures (Figure 4.13 b). In our system, we were able to statistically incorporate porphyrins and use
supramolecular peptide fibers as templates for chromophore localization through a simplistic design with equivalent efficiencies

4.4. Conclusions

In summary, we have demonstrated spontaneous formation of highly ordered supramolecular assemblies, as a result of spontaneous in-situ dipeptide cyclisation in aqueous media, resulting in a variety of supramolecular architectures (fibres, tubes, tapes). The nanostructures generated through co-assembly between c[LF] and Fe\textsuperscript{III}–TMPyP have shown the potential to function as catalysts in oxidation of pyrogallol. Cyclic dipeptides have been previously suggested as antimicrobial and antiviral agents\textsuperscript{36-37} and this study might pave the way for spontaneous fabrication of peptide nanostructures with the desired structure and function. More generally, our work is an example of chemically triggered formation of supramolecular assemblies,\textsuperscript{38} i.e. the chemical conversion of non-associating precursors to self-assembling architectures, has gained

Figure 4.13: a). Lineweaver-Burk plot on oxidation of pyrogallol by 5 mmol kg\textsuperscript{-1} c[LF] with 1 \textmu{}M Fe\textsuperscript{III}–TMPyP. and b). kinetic data calculated for 5 mmol kg\textsuperscript{-1} c[LF] - [FeIII–TMPyP].
significant interest as a means to control structural, spatial and dynamic features of supramolecular nanomaterials.\textsuperscript{39-45} A number of supramolecular functionalities have been reported by using \textit{in situ} formation of self-assembly building blocks that are difficult to achieve by using conventional self-assembly approaches, including cascade networks,\textsuperscript{46-47} localized nanostructure formation to selectively kill cancer cells,\textsuperscript{48} intracellular imaging,\textsuperscript{49} transient electronic wires,\textsuperscript{27} and gelation at the interface of biological membranes.\textsuperscript{50} More generally, the spontaneous catalytic formation of supramolecular assemblies described here involving the formation of amide bonds, might also find interest in a prebiotic manner, as the formation of diketopiperazines is considered as a probable route for prebiotic peptide formation.

4.5. Experimental

4.5.1. Materials

Aspartame (DF-OMe), LL-OMe, pyrogallol, hydrogen peroxide and meso-tetrakis-(4-N-methylpyridyl) porphyrin (TMPyP) were purchased from Sigma Aldrich and used as received. All the other dipeptide esters (LF-OMe, \textsuperscript{\textit{D}}\textsuperscript{L}\textsuperscript{D}-OMe and FF-OMe) were purchased from Genscript and CS Bio. The purity was 95\% and the dipeptide esters were used as TFA salts.

4.5.2. Methods

4.5.2.1. Sample Preparation

The concentration used for the dipeptide methyl esters (DF-OMe, LL-OMe, LF-OMe, \textsuperscript{\textit{D}}\textsuperscript{L}\textsuperscript{D}-OMe, FF-OMe) was 20 mmol kg\textsuperscript{-1}. 100 mM sodium phosphate buffer at pH 8 was used to dissolve the peptides. The samples were vortexed for 60s and sonicated for 120s in order to obtain a homogenous solution. For the co-assembly experiments, 2 mM of Fe\textsuperscript{III}-TMPyP was
mixed with 20 mmol kg\(^{-1}\)M of dipeptide methyl esters in 1 mL of 100 mM sodium phosphate buffer pH 8. The samples for catalytic activity were tested in concentration of 1, 2 and 5 mmol kg\(^{-1}\) of LF-OMe in the presence of 1 \(\mu\)M Fe\(^{III}\)-TMPyP. For each c[LF]-Fe\(^{III}\)-TMPyP hydrogel, 50 mM hydrogen peroxide was added, followed by 1 mM of pyrogallol. Oxidation product was monitored over time at \(\lambda_{max}\) (420 nm) using UV-Vis spectroscopy.

**4.5.2.2. Preparation of Fe\(^{III}\)-TMPyP**

Fe\(^{III}\)-TMPyP was prepared by a modified procedure reported by Yamaguchi et al. *Chem. Eur. J.* 2004, 10, 6179-6186. Modified procedure is as follows: 250 mg TMPyP (Obtained commercially from Sigma Aldrich) was dissolved in 100 ml water and refluxed under nitrogen atmosphere for two hours in presence of 500 molar equivalents of FeCl\(_2\).4H\(_2\)O. A visible color change from brown to green with quenching of TMPyP emission was observed. Solution was then cooled down to room temperature and 84 ml of 2 M sodium perchlorate solution was added to precipitate the product. The reaction mixture was then allowed to stand overnight in dark. The mixture was then centrifuged (10000 rpm, 10 mins, 5 °C) in aliquots. All aliquots were combined and washed with 50 ml of perchloric acid. This was done to remove excess metal ions. The paste that resulted was dried under vacuum and dissolved in acetonitrile. Fe\(^{III}\)-TMPyP readily dissolved in acetonitrile with undissolved excess metal salt (that persisted from the perchloric acid wash) left behind. Tetraethylammonium chloride was added in small aliquots to precipitate the compound. The precipitate formed is decanted and washed with chloroform to remove excess Tetraethylammonium chloride. The washed precipitate was then dried under vacuum to yield pure Fe\(^{III}\)-TMPyP. Yield =47%. HRMS: Mass calculated for Fe\(^{III}\)-TMPyP [C\(_{44}\)H\(_{38}\)FeN\(_8\)\(^{4+}\)] = 734.6211, mass obtained [C\(_{44}\)H\(_{38}\)FeN\(_8\)\(^{4+}\)-2H+OH]/4 (m/z=m/4) = 187.3110.
4.5.2.3. High Pressure Liquid Chromatography (HPLC)

A Dionex P680 HPLC system equipped with a Macherey-Nagel C18 column of 250 mm length, 4.6 mm internal diameter and 5 mm particle size was used to quantify conversions to peptide derivatives. The gradient used was a linear exchange between 20% acetonitrile in water at 2.5 min to 80% acetonitrile/water. Sample preparation involved by mixing 20 μl of the gel, solution or precipitation with 980 μl acetronitrile/water (50:50 mixture) containing 0.1% trifluoroacetic acid. Vortexing and sonication were also applied prior to sampling for the time-course experiments. The reactions were typically analyzed for up to 7 days with no significant compositional changes after 48 hours. Reaction monitoring stopped when changes were no longer observed using HPLC.

4.5.2.4. Mass spectrometry

Samples were analyzed on an LCMS system comprised of an Agilent 1200 LC system coupled to an Agilent 6340 ion trap mass spectrometer. Samples were injected onto an Agilent Zorbax column (SB-C8, 5 uM, 2.1 x 50 mm) using a linear gradient of 5-95% acetonitrile in water (1% formic acid) at a flow rate of 200 uL/min over 10 minutes.

4.5.2.5. Fourier Transform Infrared Spectroscopy

The concentration used for the FT-IR measurements was 20 mmol kg⁻¹ of the dipeptide esters at pH 8 in D₂O. The measurements were performed after the reaction was completed (72 hours). FTIR spectra were acquired in a Bruker Vertex spectrometer with a spectral resolution of 2 cm⁻¹. The measurements were performed in a standard infrared cuvette (Harrick Scientific), in which
the sample was contained between two CaF$_2$ windows (thickness, 2 mm) separated by a 27 mm polytetrafluoroethylene spacer.

**4.5.2.6. Circular Dichroism Spectroscopy (CD)**

Samples were pipetted into a 0.1 mm cell and spectra were measured on a Jasco J600 spectropolarimeter with 1s integrations, a step size of 1 nm and a single acquisition with a slit width of 1 nm. The measurements were performed after the reaction was completed (72 hours).

**4.5.2.7. Atomic Force Microscopy (AFM)**

Samples were prepared by drop casting 20 μL of each sample onto a freshly cleaved mica substrate (G250–2 Mica sheets 1”× 1 ”× 0.006” (Agar Scientific Ltd)) attached to an AFM support stub and left to air dry overnight in a dust-free environment, prior to imaging. The images were obtained by scanning the mica surface in air under ambient conditions using a Multimode 8 FastScan Microscope (Bruker) operated in scan assist air mode with a fastscan B tip. The AFM scans were taken at a resolution of 512 × 512 pixels. The images were analyzed using NanoScope Analysis software Version 1.40.

**4.5.2.8. Rheology**

Rheological properties of c[LF] (5, 10, 20 and 40 mmol kg$^{-1}$) were assessed using an Anton Paar Physica MCR 101 rheometer with temperature controlled at 25 °C using a 10 mm vane geometry. Samples were prepared using a mold with 300 ul from each concentration. Amplitude sweeps were performed to obtain the strain for each sample to run the frequency sweeps. To ensure the measurements were made in the linear viscoelastic regime, amplitude sweeps were
performed at constant frequency of 1 Hz, from shear strain 0.01–100%. Gel formation was the monitored by measuring $G'$ and $G''$ at 0.1 to 100 Hz frequency range using the shear strain for each sample.

The self-healing measurements were taken for the 20 mmol kg$^{-1}$ c[LF] hydrogel with a 0.03% strain for 120 seconds and 120 sec for healing. This a was continued for six cycles. For the temperature sweep, a temperature ramp from 25 °C- 70 °C was employed under 0.03% with 20 mmol kg$^{-1}$ c[LF] hydrogel.

**4.5.2.9. UV-VIS Spectroscopy**

1ml samples were prepared in 10mm quartz cuvette. UV-VIS absorbance spectra were recorded with a Jasco V-660 spectrophotometer at a medium scanning speed of 100 nm min$^{-1}$. Time dependent measurements were collected at 420 nm wavelength.

**4.5.2.10. Transmission electron microscopy**

Carbon-coated grids were purchased from Electron Microscopy Sciences. A drop (5 µl) of the sample solution was applied to the carbon-coated grid and incubated for one minute. Excess solution was removed by blotting the grid with a piece of filter paper, followed by staining with 5 µl of 2% (w/v) uranyl acetate solution for 30 seconds. After blotting excess stain solution, the grid was left to air dry. The negatively stained sample was imaged in FEI TITAN Halo TEM operating at 300 kV. Images were recorded in the low-dose mode (20 e− Å$^{-2}$) on an FEI CETA 16M camera (4,096 × 4,096 pixels).

**4.6. References**


5. Sequence Dependent Tripeptide/porphyrin Co-assembly in Aqueous Medium

Manuscript in preparation. Nadeesha K. Wijerathne, Mohit Kumar, Ankit Jain and Rein V. Ulijn,

Chemical Communications
5.1. Introduction

Peptide nanotechnology has been extensively studied over last decade due to a number of attractive features allied with peptides.\textsuperscript{1-2} They exhibit wide-ranging structural and functional diversity arising from its primary building blocks, the 20 gene-coded amino acids. Short peptides combine some of the advantages synthetic chemical systems and proteins, in that they are functionally versatile, biocompatible while also available at large scale\textsuperscript{3-4} as summarized in the literature review (Chapter 2).\textsuperscript{5-6} These materials have current and potential applications in various fields, including health care, biosensing and energy harvesting.\textsuperscript{7-9} In this chapter we investigate the co-assembly of tripeptides with porphyrins to obtain energy transfer materials with potential future applications in light harvesting.

Previous reports have demonstrated that tripeptides are versatile building blocks for the design of functional nanoscale materials, covering 8000 possible combinations cover a large chemical space.\textsuperscript{10} Ulijn, Tuttle and co-workers have previously studied and reported design rules on the sequence order of amino acids by computationally screening all 8000 possible tripeptide combinations.\textsuperscript{11} They reported four hydrogel forming unprotected tripeptides namely KYF, KFF, KYY and KYW that self-assembled into fibrous nanostructures upon adjusting the pH to 7.5 in water. The formation of stable emulsions in oil/water interface including KYF, KFF, FFD and DFF, demonstrated that the cationic and anionic peptides exhibit different stabilities in emulsions formed depending on the sequence and temperature.\textsuperscript{12} Furthermore, sequence isomers of tripeptides could be used as tunable precursors with varying degrees of supramolecular order/disorderd depending on the peptide sequence. These peptides could be used to produce polymeric pigments that are close mimics of the biopolymer melanin, by using six sequences of isomeric tripeptide in controlled oxidation.\textsuperscript{13} Co-assembly of functional molecules into peptide
matrices could introduce new properties with extended order supported by peptide supramolecular structures. Selective complex formation of co-assembled tripeptide has demonstrated utilizing a structure forming tripeptides (FFD and DFF) and a Cu binding GHK for the co-assembly showing that the non-gelator GHK transformed into a hydrogel upon addition of an equimolar solution of CuCl₂.

Peptides were previously shown to be suitable building blocks for the assembly of supramolecular peptide/porphyrin structures for the design of photosensitive materials with applications in light harvesting, energy transfer, catalysis and oxygen storage. Porphyrin/peptide co-assembly by means of short peptides is important due to the flexible connections governed by non-covalent bonding including π-π stacking interactions, hydrogen bonding, van-der waals forces and electrostatic interactions.

There are only few reports on the use of peptide/porphyrin co-assemblies in aqueous media, while this would be useful for several applications that requires non-organic environment at physiological pH. The challenge of assembly of hydrophobic structures such as porphyrins in water is associated with the formation of optimized nanostructures is the need of well-defined control over the supramolecular organization, requiring precise control over temperature and pH to avoid the unwanted aggregate formation of porphyrin, with previous reports using acidic conditions (pH<2) or organic co-solvents to induce self-assembly. Metalloporphyrin monomers have also been studied as catalysts in a variety of oxidation reactions such as epoxidation of olefins, alkane hydroxylation, hydrodehalagenation of organic halides and peroxidase like catalytic activity in small organic molecules. It has been shown that the catalytic activity of metalloporphyrin is enhanced when supported by a scaffold such as peptides.
Herein we demonstrate the hydrogel forming co-assembly of charged free base porphyrins and a metalloporphyrin into a range of closely related cationic tripeptide scaffolds in aqueous medium at the physiological pH exhibiting a sequence dependent co-assembly and energy transfer potential (Scheme 5.1.).

Scheme 5.1: Chemical structures of a). peptides (i) KFF (ii) KYW (iii) KYF (iv) KYY b). porphyrin (i) TPPS (ii) TMPyP c). schematic diagram of peptide-porphyrin co-assembly
5.2. Objectives

The sequence of four cationic peptides previously reported to self-assemble into nanofibers and two charged porphyrins were utilized to study different co-assembling propensities. Based on the hypothesis that the cationic peptide will have an interaction with anionic porphyrin and cationic porphyrin to interact with peptides via deprotonated C terminus, we set two main objectives.

i. To study the effect of primary sequence of the peptide to co-assemble porphyrin molecules inside the peptide nanofibers.

ii. To investigate the energy transfer efficiency between the aromatic residues and porphyrin molecules in the peptide-porphyrin co-assembled nanostructures.

5.3. Results and Discussion

5.3.1. Preparation of Self-Assembled Tri-peptide Nanostructures and Co-assembly of Tri-peptide-Porphyrin Nanostructures

The porphyrin library consisted of an anionic porphyrin, TPPS (4,4′,4,4″,4″′-Porphine-5,10,15,20-tetrayl)tetrakis(benzenesulfonic acid)) to induce electrostatic interaction with lysine residue and a cationic porphyrin, TMPyP (5,10,15,20-Tetra(4-pyridyl)-21H,23H-porphine) and a metalloporphyrin FeIII-TMPyP (Iron (III) (5,10,15,20-Tetra(4-pyridyl)-21H,23H-porphine)), to interact with negatively charged terminal COO⁻ (Scheme 5.1.b.).

KYF, KYY, KFF and KYW has been previously studied and already known fibrous nanostructures stabilized by β-sheet-like hydrogen bonding patterns (Scheme 5.1.a.). It was previously reported that the optimized concentration for the self-assembly of tri-peptide molecules is 40 mM in distilled water at pH 7.5.¹² A porphyrin solution of 0.5 mM was introduced to the 40 mM peptide solution and adjusted the pH to 7.5 using 0.5 M NaOH to
facilitate the supramolecular structure formation. The peptide-porphyrin hydrogels formed after adjusting the pH to 7.5 exhibited an intrinsic color, which corresponds to color of the porphyrin, used. (Figure 5.1.)

![Figure 5.1: Photographic images of hydrogels formed after self-assembly of top panel: peptides and co-assembly of middle panel: peptide-TPPS and bottom panel: peptide-TMPyP (40 mM peptide-0.5 mM porphyrin)](image)

5.3.2. Atomic Force Microscopy Analysis

The morphology of the porphyrin co-assembled nanostructures was explored by the Atomic Force Microscopic (AFM) imaging and compared to that of peptide fibers without porphyrin (Figure 5.2.). The peptide nanofibers were more tangled and becomes wider but shorter in the presence of porphyrin, except for KFF in the presence of TPPS. The TPPS incorporated KFF nanofibers exhibited thinner but longer fibers, that were less tangled. It was observed that the introduction of porphyrin molecules into the peptide nanostructures significantly changes the
morphology of the peptide fibers indicating the co-assembly and/or self-aggregation of porphyrins which is further investigated by absorbance (section 5.3.3.) and emission spectroscopy (section 5.3.4) followed by confocal imaging (section 5.2.6.)

![AFM images of hydrogels](image)

**Figure 5.2:** AFM images of hydrogels of top pannel: peptide only, middle panel: peptid-TPPS and bottom panel peptide-TMPyP (40 mM peptide-0.5 mM porphyrin) scale bar 250 nm

### 5.3.3. Absorbance Spectroscopy Analysis

A detailed absorbance spectroscopic study was carried out to examine the self-aggregating and co-assembling propensity of porphyrin molecules inside the nanofibers (**Figure 5.3**). TPPS is known to produce self-aggregates and self-assemble into fibers at the low pH values, hence it is important to ensure that the porphyrin molecules are presented in their monomeric state to get incorporated into the peptide nanofibers.²⁹
Absorbance of peptide hydrogels was measured before and after incorporation of porphyrin molecules compared with free porphyrin. The changes in absorbance upon TMPyP incorporation are almost negligible compared to the free porphyrin molecules, where no significant peak shift or broadening was observed following the incorporation (Figure 5.3.b). On the other hand, TPPS absorbance spectra exhibited changes in the Soret-band position and width upon co-assembly. A significant band broadening was observed for all four peptides, with different shifts in the Soret-band for each peptide. Remarkably, in the presence of KYF, the peak is blue-shifted, which indicates the formation of H-type self-aggregates. By contrast, in the presence of KYW the porphyrin exhibited a red-shift, which indicates the formation of J-type aggregates. KFF and KYY didn’t show a noteworthy spectral shift, beyond a slight red-shift compared to free TPPS. It was noted that for all peptides studied, the presence of two different aromatic residues in the tripeptide sequence induces the self-aggregation of TPPS into different arrangements where phenylalanine appears to induce the formation of H aggregates and tryptophan organize porphyrins in J aggregates due to differences in pi-stacking interactions. The two peptides with homo-dyad peptides did not influence the aggregation of porphyrin.

Figure 5.3: Absorbance spectra comparison of peptide-porphyrin nanostructures in the presence of a). TPPS b). TMPyP and c). Fe^{III}TMPyP (40 mM peptide-0.5 mM porphyrin)
5.3.4. Emission Spectroscopy Analysis

Since the absorbance studies of TPPS incorporated peptides indicated a self-aggregation of porphyrin, the fluorescence spectroscopy was carried out to investigate the presence of monomeric porphyrin inside peptide nanofibers. A strong emission signal in the porphyrin region indicated the presence of monomeric porphyrin inside the peptide nanofibers, hence potential co-assembly with both TPPS (Figure 5.4.a) and TMPyP (Figure 5.4.b). The amount of porphyrin monomers presents inside each peptide scaffolds was observed to be different depending on the emission intensity of porphyrin in the presence of each peptide. KYW indicated a lowest emission followed by KYF in the presence of TPPS. This observation correlates with the absorbance spectroscopic results confirming that amount of monomeric porphyrin present inside the KYW and KYF hydrogels are lower compared to KFF and KYY due to the self-aggregation. TMPyP incorporated hydrogels indicated a decrease in emission intensity depending on the hydrophobicity of the peptide. The order of increasing emission intensity was

![Fluorescence spectra](attachment:image.png)

**Figure 5.4:** Fluorescence spectra of peptide-porphyrin co-assembled hydrogels with a). TPPS and b). TMPyP (40 mM peptide-0.5 mM porphyrin)
KYY<KYF<KYW<KFF demonstrating more hydrophobic peptides show higher emission compared to hydrophilic peptides. It can be concluded from these observations that the hydrophobic peptides support the co-assembly of TMPyP than hydrophilic peptides.

Conclusively, anionic TPPS tend to form self-aggregated and also get co-assembled into the peptide matrix as monomers, while cationic TMPyP gets co-assembled without any aggregation. This observation indicates that the sequence of the peptide and the charge of the porphyrin are important factors in dictating the peptide induced co-assembling or self-assembling of porphyrin and peptide molecules.

![Image of peptide nanostructures](image)

**Figure 5.6:** Integrated Confocal microscopic and bright field images of tri-peptide nanostructures in the absence of porphyrin

### 5.3.5. Confocal Microscopy Analysis

Confocal microscopic imaging was carried out to further confirm incorporation of TPPS into the peptide fibers by exciting the peptide-porphyrin hydrogels at 405 nm (in the porphyrin region) and collecting from 600-700 nm (figure 5). The porphyrin region was selected to make sure the observed emission is only arising from the porphyrin molecules inside the hydrogels (**Figure 5.5**). KYF exhibited an ordered fiber network with porphyrin emission, while KFF showed thin
long fibers. KYY formed of short thin fibers and peptide-incorporated KYW showed a densely packed morphology where individual fibers cannot be distinguished.

As a control, all four tri-peptide hydrogels were analyzed without porphyrin by exciting in the porphyrin region. As expectedly, no emission was detected for the hydrogels without porphyrin confirming that the emission is exclusively originating from porphyrin (Figure 5.6). Free porphyrin sample was also imaged at the pH of interest (pH 7.5) to investigate the generation of self-aggregates. There was no aggregation observed in the free TPPS indicating the absence of self-aggregation at physiological pH.

Figure 5.5: Confocal microscopic image comparison of TPPS co-assembled nanostructures and free TPPS (40 mM peptide-0.5 mM porphyrin) scale bar 5μM

5.3.6. Fourier Transform Infrared Spectroscopy analysis

FTIR analysis was employed to investigate the hydrogen bond formation in the tripeptide/porphyrin co-assembled nanofibers. The tripeptide supramolecular structures form a salt-bridge by head to tail interaction of the COO⁻ and H₃N⁺ termini and hydrogen bonding between amino acid residues in the peptide side chain. The salt bridge formation can be identified by the shifting of the peak from 1560 cm⁻¹ (that corresponds to the deprotonated COO⁻ group) to a higher wavenumber (1580 cm⁻¹) and peaks around 1625 cm⁻¹ and 1650 cm⁻¹ confirms the hydrogen bond formation (Figure 5.7).³⁶ Incorporation of TPPS into the nanofiber network gave rise to changes in hydrogen bonding and salt bridge formation. After introducing negatively
charged TPPS it may be expected to interact either with positively charged lysine side chain, or protonated H$_3$N$^+$ terminus, or both. We can suggest that the porphyrin molecules are more likely to have an interaction with lysine residue rather than the positively charged N-terminus since the salt bridge (1580 cm$^{-1}$) has been kept intact after co-assembly with KYF, KYW (Figure 5.7.b and c and d). In contrast, KYY has almost lost the signal in the presence of TPPS (Figure 5.7.d) and KFF starting to show a salt-bridge formation in the presence of TPPS (Figure 5.7.a). There is a change in the hydrogen bonding between KYY molecules after TPPS 1560 cm$^{-1}$ (that corresponds to the deprotonated COO$^-$ group) to a higher wavenumber and peaks around 1625 cm$^{-1}$ and 1650 cm$^{-1}$ confirms the hydrogen bond formation (Figure 5.7). These results may suggest that the porphyrin molecules are more likely to have an interaction with lysine residue rather than the positively charged N-terminus since the salt bridge (peak around 1580 cm$^{-1}$) has been kept intact after co-assembly with KYF, KYW (Figure 5.8. b and c). KYY has almost lost the signal in the presence of TPPS (Figure 5.8.d) and KFF starting to show a salt-bridge formation in the presence of TPPS (Figure 5.8.a). There is a change in the hydrogen bonding

![Figure 5.7: Comparison of FTIR spectra between tri-peptides and tri-peptide-porphyrin nanostructures. (40 mM peptide-0.5 mM porphyrin)](image)

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between KYY molecules after TPPS incorporation while KYF, KYW and KFF have maintained the H-bonding interactions in the presence of TPPS (1625 cm\(^{-1}\) and 1650 cm\(^{-1}\)).

In the presence of cationic porphyrin TMPyP, KYF and KYW maintain both hydrogen bonding and the salt-bridge as evident by peaks at 1625 cm\(^{-1}\) and 1650 cm\(^{-1}\) and 1580 cm\(^{-1}\) respectively. (Figure 5.9.b and c) while KFF is showing a disruption in hydrogen bond formation (Figure 5.8.a). On the other hand, KYY exhibit intact hydrogen bonding between the residues but changes in salt-bridge formation (Figure 5.8.d). We can expect for KFF to have hydrophobic interactions through its fibrous core, as the most hydrophobic peptide in the list. It can be concluded that in the presence of KYF and KYW, the porphyrin-peptide interactions are insufficiently strong to disrupt either the salt-bridge or the hydrogen bonds while, for KYY gets its hydrogen bonding patterns disrupted by anionic porphyrin. (Figure 5.10). Insufficiently strong to disrupt either the salt-bridge or the hydrogen bonds while, for KYY gets its hydrogen bonding patterns disrupted by anionic porphyrin. (Figure 5.10).

5.3.7. Circular Dichroism Analysis

In order to understand the supramolecular chirality in porphyrin incorporated peptide nanofibers, Circular Dichroism (CD) analysis was carried out and compared with peptide free peptide fibers (Figure 5.8. and Figure 5.9.). The structural changes in the peptide supramolecular assemblies can be monitored by analyzing the amide I region before and after the co-assembly of porphyrin and the helical induction of porphyrin molecules was examined by studying the porphyrin region after incorporation. Changes in the supramolecular structures of KYF, KFF and KYY were
observed in the presence of TPPS, but not for KYW (Figure 5.8.a). It was observed that the most polar KYF, and KYY are starting to lose the antiparallel β-sheet characteristic after the incorporation of TPPS into the peptide nanofibers while KFF exhibited an enhanced chirality compared to that of KFF without porphyrin incorporation. KYW has shown a loss in the degree of chiral induction in the presence of TPPS. After the co-assembly of TMPyP, KYF, KFF and KYY showed a deviation from the intrinsic β-sheet structure and latter two exhibited an enhancement of chirality in the presence of porphyrin. On the other hand, KYW didn’t show a change chiral induction in the amide region (190-300 nm) after the incorporation of porphyrin (Figure 5.9.a). There was no contribution to the signal in the porphyrin region from TMPyP with any of the peptides (Figure 5.8.b and 5.9.b.). This can be attributed to the absence of self-aggregation with TMPyP. On the other hand, KYF and KFF showed a chiral induction in the porphyrin.
Figure 5.8: CD analysis of tripeptide-TPPS co-assembled nanostructures
a). amide I region b). porphyrin region (40 mM peptide-0.5 mM porphyrin)
**Figure 5.9:** CD analysis of tripeptide-TMPyP and Fe^{III}-TMPyP co-assembled nanostructures a). amide I region b). porphyrin region (40 mM peptide-0.5 mM porphyrin)
To summarize the observations, KYW nanofibers with TPPS tends to lose the chiral induction but maintained it with TMPyP after incorporation of porphyrins while KYF and KYY were starting loose the β-sheet conformation compared to that in peptide nanofibers. KFF has enhanced the chiral induction in the presence of porphyrin compared to that of unincorporated fibers. Following the observations, it can conclude that the co-assembly of porphyrin molecules leads to changes in initial supramolecular structure of self-assembled peptides and the degree of chirality change depends upon the tri-peptide sequence used. Introduction of tryptophan (W) in the c-terminus has a direct effect on reducing the chirality with anionic porphyrins while having two neighboring phenylalanine residues enhanced the chiral induction after incorporation

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<tr>
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<td>KYY</td>
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<td>↓</td>
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<tr>
<td>TMPyP</td>
<td>↑</td>
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<tr>
<td>Fe\textsuperscript{III}-TMPyP</td>
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**Figure 5.10:** Summary of FTIR and CD data of peptide-porphyrin co-assemblies compared to peptides

- ●: no change
- ↑: increase
- ↓: decrease
- ||: change of direction in CD

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5.3.8. Energy Transfer Efficiency Calculations Between Aromatic Residues and Porphyrin

Fluorescence spectroscopy can be used to investigate the interaction between peptide and porphyrin in supramolecular assemblies. It can, in turn, be utilized to study the potential of energy transfer between peptides and porphyrins. Moreover, a thorough energy transfer probing will provide an explanation about the proximity of donor (peptide) and acceptor (porphyrin) molecules, as there are a few conditions that must be satisfied for Förster Resonance Energy Transfer (FRET) to occur.\[^{37}\] In order to achieve FRET emission spectra of donor molecules must overlap with the absorption spectra of acceptor molecules. Furthermore, the distance between the donor and acceptor has to be maintained in the range of 1 nm-10 nm. Due to its sensitivity to distance, we can obtain an idea about the closeness of donor-acceptor molecules in the supramolecular assemblies depending on their ability to participate in FRET.

Following that, emission spectra for peptide-porphyrin hydrogels were collected upon excitation at 285 nm (tyrosine absorbance band) from 350nm to 800 nm with KYF, KYY and KYW in the presence of TPPS and TMPyP separately (Figure 5.11.a and b.). A broad emission band around 450 nm was detected for tyrosine that overlaps with the absorption band of porphyrin. There was an extra band observed in the porphyrin region from 600 nm to 800 nm with KYF and KYY upon tyrosine emission. This observation can be attributed to the energy transfer between tyrosine residue in peptide and porphyrin molecules that has been incorporated into the peptide fiber network. For the tyrosine free peptide KFF, emission was collected by exciting phenylalanine residue at 255 nm. The excitation spectra were collected for all the hydrogel samples for peptide region (200-350 nm) containing TSSP and TMPyP by measuring the
emission of the samples at 655 nm (porphyrin region) and was compared with the absorbance spectra of respective hydrogel samples at peptide region (200-350 nm).

Control measurements were done using tripeptides in the absence of porphyrin, as well as porphyrin in the absence of peptide. Upon emission at 275 nm, a significantly lower emission band compared to that of KYF-porphyrin nanostructures was observed with both TPPS and TMPyP solutions. The emission was slightly quenched inside KFF-porphyrin and KYY-porphyrin nanostructures in the presence of TPPS but increased emission in the presence of TMPyP. KYW emission was detected to be scientifically lower with both porphyrins. When

![Figure 5.11](image)

**Figure 5.11**: Fluorescence analysis of tri-peptide–porphyrin nanostructures with a) TPPS b).TMPyP and c). tripeptides only showing the energy transfer (40 mM peptide-0.5 mM porphyrin).

peptide solutions were excited at 275 nm (KYF, KYY and KYW) and 255 nm (KFF), no emission was detected in the porphyrin region (**Figure 5.11.c**).

The energy transfer efficiency percentages were calculated for each peptide in the presence of porphyrin (Table 1). KYF and KFF exhibited higher energy transfer efficiency in the presence of anionic TPPS than that of cationic TMPyP. KFF displayed a moderate energy transfer with both porphyrins whereas KYF showed the least efficiency. By contrast, KYY didn’t exhibit efficient energy transfer in the presence of anionic TPPS yet showed enhanced energy transfer with
TMPyP even though a smaller number of monomeric porphyrins are available inside the hydrogel that observed from from fluorescence spectroscopy. It might be due to the better alignment of porphyrin molecules inside the peptide fibers of KYY. Following the observation from absorbance fluorescence and confocal imaging analysis KYW didn’t display any type of energy transfer with both TPPS and TMPyP. We can clearly observe that the energy transfer efficiencies depend upon the primary sequence of the tri-peptides hence the organization of porphyrin molecules inside the peptide scaffolds can be controlled by the peptide sequence. KFF being the most hydrophobic peptide among the four, capable of supporting the organization of both anionic and cationic porphyrins in such a way that they fulfill the requirements for FRET by maintaining electrostatic and hydrophobic interactions.

It can be concluded that the peptide supramolecular assembles supports the organization of porphyrin to facilitate efficient energy transfer between aromatic peptide residues and porphyrin molecules and the generated peptide–porphyrin nanostructures will pave the path to design devices to harvest light in the UV range of electromagnetic spectrum.

Table 5.1: Percent energy transfer efficiencies between tripeptide and porphyrin empty cells indicate no energy transfer was detected)

<table>
<thead>
<tr>
<th></th>
<th>KFF</th>
<th>KYW</th>
<th>KYF</th>
<th>KYY</th>
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<tbody>
<tr>
<td>TPPS</td>
<td>40.31</td>
<td>-</td>
<td>30.21</td>
<td>-</td>
</tr>
<tr>
<td>TMPyP</td>
<td>36.34</td>
<td>-</td>
<td>23.79</td>
<td>88.31</td>
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5.3.9. Peptide-Metalloporphyrin Co-assembly

Finally, the ability to co-assemble a metalloporphyrin into the tri-peptide nanostructures were investigated using Fe\textsuperscript{III}-TMPyP. Hydrogels with an intrinsic color corresponding to Fe\textsuperscript{III}-TMPyP was formed after incorporation and the AFM images indicated that fibrous nanostructures were formed (Figure 5.12.).

Absorbance of Fe\textsuperscript{III}-TMPyP was measured before and after co-assembly and no change in the absorbance was detected after incorporation indicating the occurrence of monomeric state of Fe\textsuperscript{III}-TMPyP in the peptide-porphyrin assemblies. This also proves absence of unwanted self-aggregates by porphyrin molecules in co-assembled system (Figure 5.3.c). FTIR analysis showed that the hydrogen bonding between peptide β-sheets kept intact upon co-assembly, however the salt-bridge has slightly disturbed in the presence of porphyrin. Following the above observations, we can conclude that the Fe\textsuperscript{III}-TMPyP molecules are being co-assembled into the peptide nanofibers (Figure 5.13.).
We have studied the co-assembly of charged porphyrins with four closely related self-assembling tripeptides to assess the effect of substitution of aromatic amino acids (F, Y, W) on structure and consequent energy transfer. It was observed that the co-assembled nanostructure formation depends strongly upon the sequence of the peptide used as well as the charge of the porphyrin. The hydrogen bonding patterns and the helical induction inside the tripeptide fibers exhibited changes, with the hydrophilic peptide KYY showing a disruption of hydrogen bond formation in the presence of anionic porphyrins and the most hydrophobic peptide KFF showing an enhancement in the salt-bridge formation in the presence of both anionic and cationic porphyrins.

**Figure 5.13:** FTIR spectrum of tripeptide-Fe\textsuperscript{III}-TMPyP co-assembled nanostructures compared to peptides (40 mM peptide-0.5 mM porphyrin)

### 5.4. Conclusions

We have studied the co-assembly of charged porphyrins with four closely related self-assembling tripeptides to assess the effect of substitution of aromatic amino acids (F, Y, W) on structure and consequent energy transfer. It was observed that the co-assembled nanostructure formation depends strongly upon the sequence of the peptide used as well as the charge of the porphyrin. The hydrogen bonding patterns and the helical induction inside the tripeptide fibers exhibited changes, with the hydrophilic peptide KYY showing a disruption of hydrogen bond formation in the presence of anionic porphyrins and the most hydrophobic peptide KFF showing an enhancement in the salt-bridge formation in the presence of both anionic and cationic porphyrins.
leading to different organization of porphyrin molecules inside the peptide matrix when they get co-assembled. The FRET efficiencies varied drastically with the sequence of the peptides as well as the charge of the porphyrin employed confirming the observations from FTIR analysis. With the disturbed hydrogen bonding after co-assembly with anionic porphyrins, KYY didn’t exhibit any transfer between aromatic residues lacking the proper alignment of porphyrins in the peptide nanofibers. KFF being the most hydrophobic peptide shows adequate organization with both cationic and anionic demonstrating the importance of hydrophobic interactions between porphyrin and peptides. KYW couldn’t achieve the proper alignment of porphyrin, hence didn’t display an efficient energy transfer. These differential organizations resulted in fundamentally different stacking arrangements. Conclusively, it was proved that the organization of charged porphyrins can be controlled by small changes in the aromatic dyad sequence of the peptide employed in co-assembly.

5.5. Experimental

5.5.1. Materials

4,4′,4,4″,4‴-(Porphine-5,10,15,20-tetrayl)tetrakis(benzenesulfonic acid) (TPPS) and mesotetakis-(4-N-methylpyridyl) porphyrin (TMPyP) were purchased from Sigma Aldrich and used as received. All the other tripeptides (KYF, KFF, KYY and KYW) were purchased from Genscript. The purity was 98% and the tripeptides were used as TFA salts.

5.5.2. Methods

5.5.2.1. Sample Preparation

The concentration used for the tripeptides (KYF, KFF, KYY and KYW) was 20 mM. Peptides were dissolved in deionized water and the pH was adjusted to 7.5 using 0.5 M NaOH. The samples were vortexed for 60s and sonicated for 120s in order to obtain a homogenous solution.
For the co-assembly experiments, 0.5 mM of porphyrins (TPPS, TMPyP, Fe\textsuperscript{III}-TMPyP) was mixed with 20 mM of tripeptide in deionized water and the pH was adjusted to 7.5 using 0.5 M NaOH.

5.5.2.2. Preparation of Fe\textsuperscript{III}-TMPyP

Fe\textsuperscript{III}-TMPyP was prepared by a modified procedure reported by Yamaguchi et al. Chem. Eur. J. 2004, 10, 6179-6186. Modified procedure is as follows: 250 mg TMPyP (Obtained commercially from Sigma Aldrich) was dissolved in 100 ml water and refluxed under nitrogen atmosphere for two hours in presence of 500 molar equivalents of FeCl\textsubscript{2}.4H\textsubscript{2}O. A visible color change from brown to green with quenching of TMPyP emission was observed. Solution was then cooled down to room temperature and 84 ml of 2 M sodium perchlorate solution was added to precipitate the product. The reaction mixture was then allowed to stand overnight in dark. The mixture was then centrifuged (10000 rpm, 10 mins, 5 °C) in aliquots. All aliquots were combined and washed with 50 ml of perchloric acid. This was done to remove excess metal ions. The paste that resulted was dried under vacuum and dissolved in acetonitrile. Fe\textsuperscript{III}-TMPyP readily dissolved in acetonitrile with undissolved excess metal salt (that persisted from the perchloric acid wash) left behind. Tetraethylammonium chloride was added in small aliquots to precipitate the compound. The precipitate formed is decanted and washed with chloroform to remove excess Tetraethylammonium chloride. The washed precipitate was then dried under vacuum to yield pure Fe\textsuperscript{III}-TMPyP. Yield =47%. HRMS: Mass calculated for Fe\textsuperscript{III}-TMPyP [C\textsubscript{44}H\textsubscript{38}FeN\textsubscript{8}\textsuperscript{4+}] = 734.6211, mass obtained [C\textsubscript{44}H\textsubscript{38}FeN\textsubscript{8}\textsuperscript{4+}-2H+OH]/4 (m/z=m/4) = 187.3110.
5.5.2.3. Fourier Transform Infrared Spectroscopy

The concentration used for the FT-IR measurements was 20 mM of the tripeptides and tripeptide/porphyrin mixtures at in D$_2$O and pD was adjusted using 0.5 M deuterated NaOH. The measurements were performed after the self-assembly was completed (24 hours). FTIR spectra were acquired in a Bruker Vertex spectrometer with a spectral resolution of 2 cm$^{-1}$. The measurements were performed in a standard infrared cuvette (Harrick Scientific), in which the sample was contained between two CaF$_2$ windows (thickness, 2 mm) separated by a 27 mm polytetrafluoroethylene spacer.

5.5.2.4. Circular Dichroism Spectroscopy (CD)

Samples were pipetted into a 0.01 mm cell and spectra were measured on a Jasco J600 spectropolarimeter with 1s integrations, a step size of 1 nm and a single acquisition with a slit width of 1 nm. The measurements were performed after the self-assembly was completed (24 hours).

5.5.2.5. Atomic Force Microscopy (AFM)

Samples were prepared by drop casting 20 µL of each sample onto a freshly cleaved mica substrate (G250–2 Mica sheets 1”× 1”× 0.006” (Agar Scientific Ltd)) attached to an AFM support stub and left to air dry overnight in a dust-free environment, prior to imaging. The images were obtained by scanning the mica surface in air under ambient conditions using a Multimode 8 FastScan Microscope (Bruker) operated in scan assist air mode with a fastscan B tip. The AFM scans were taken at a resolution of 512 × 512 pixels. The images were analyzed using NanoScope Analysis software Version 1.40.
5.5.2.6. UV-VIS Spectroscopy

20 µL samples were prepared in 0.01 mm quartz cuvette. UV-VIS absorbance spectra were recorded with a Jasco V-660 spectrophotometer at a medium scanning speed of 100 nm min⁻¹.

5.6. References


6. Conclusions and Future Directions
6.1. Conclusions

Supramolecular structures generated via self-assembly of short peptide have shown the structural and functional properties close to complex proteins. The different types of self-assembled nanoarchitectures can be obtained by simply varying the amino acid sequence of the peptide. These peptide nanoarchitectures can provide a platform to co-assemble porphyrin molecules in such a way that they can enhance their functional properties such as light harvesting and catalysis.

In particular, we have focused on reversible biocatalytic co-assembly of Fmoc based dipeptide and porphyrin molecules to generate an energy transfer hydrogel (chapter 3). The advantage of such a system is that they can provide the thermodynamically optimized structure with better organization of porphyrin molecules in the peptide matrix. It was observed that the peptide to porphyrin molar ratio can dictate the cooperative assembly vs. self-aggregation of porphyrin molecules in side the peptide fibers, thus proving the control of the positioning of porphyrin molecules. The optimized alignment of porphyrin molecules by means of peptides facilitate the efficient energy transfer between Fmoc moiety and porphyrin molecules.

In situ cyclization of liners dipeptide methyl esters in aqueous medium was also investigated with different combination of amino acids to understand the sequence dependency on self-assembly (chapter 4). Different nanoarchitectures were observed by simply changing the dipeptide sequence of peptide. The hydrogel forming cyclic assemblies also shown the tunable mechanical properties by varying the concentration of the starting material. It was also observed that the cyclic peptide hydrogels can utilized as a scaffold to co-assemble a metalloporphyrin to obtain a system with enhanced catalytic activity to oxidize small organic molecules like pyrogallol.
The organization of porphyrin molecules into a tripeptide hydrogel that is controlled by the sequence of peptide is studied with a library of cationic tripeptide and two oppositely charged porphyrins (chapter 5). The peptide induced self-aggregation and peptide mediated co-assembly of porphyrin molecules into peptide nanostructures was observed to be depending on the sequence of the tripeptide and also the charge of the porphyrin molecule. The percentage of efficient energy transfer is also dictated by the sequence of the peptide by aligning the porphyrin molecules in different distances inside the peptide assemblies.

6.2. Future Directions

From the conclusions derived from the projects presented in this thesis, there several interesting potential applications that can be suggested as future directions.

The work presented in this thesis is mainly based on the peptide mediated co-assembly of porphyrins in the aqueous medium in the physiological pH. Although the peptide-porphyrin self-assembly is extensively studied in different media such as organic solvents, less studies are done in the aqueous medium due to the high hydrophobic nature of the porphyrins. Furthermore, low pH has also utilized to promote the assembly in acidic medium. The use of traces of toxic organic solvents will limit the use of peptide-porphyrin assemblies under biological conditions specially in the medicinal chemistry applications due to the high toxicity. Most of the biological systems functions close to physiological pH. The peptide-porphyrin assemblies generated in phycological pH with aqueous medium will provide ideal conditions to mimic the biological systems in nature. The well-ordered peptide-porphyrin co-assembled hydrogels will offer a biocompatible light harvesting material for future photovoltaic devices. The ability of the hydrogel system to absorb a
broadband of electromagnetic spectrum will pave the way to generated photosensitive materials that can harvest both ultraviolet and visible range of the electromagnetic spectrum.

Furthermore, cyclic dipeptides have previously utilized as antimicrobial and antiviral agents. The self-assembly pathway in the study might open the way for spontaneous fabrication of peptide nanostructures with the desired structure and function with chemically triggered supramolecular structure formation. The in-situ formation of self-assembling building blocks can also be employed in studies where the purpose cannot be achieved conventional self-assembly approaches, including cascade networks, localized nanostructure formation to selectively kill cancer cells, intracellular imaging, transient electronic wires, and gelation at the interface of biological membranes. These systems can also utilize to form prebiotic peptide where diketopiperazine ring formation is used as one potential synthetic route.

Finally, taking the advantage of peptide-metalloporphyrin nanostructures formed with tripeptides, we are proposing the hydrogels as a catalyst for hydrodehalogenation of small organic molecules. Metalloporphyrins monomers have previously known to catalyze the dehalogenation reaction of small organic molecules in the presence of a hydrophobic medium. We are hypothesizing that the porphyrin molecules that are being incorporated into the hydrophobic core of the peptide nanofibers will be able to provide phase separation required for the catalytic reaction. On the other hand, metalloporphyrin incorporated peptide-based hydrogels can also demonstrate peroxidase-like activity in oxidizing small organic molecules.