Effect of Anions on Nanofiber Formation of $\beta$-sheet Propensity Amphiphile Peptide

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Abstract. Peptide self-assembly forms different nanostructures under simple alteration in the solution environment. Understanding the mechanism of the assembly will help us to control and tailor functional nanomaterials. This study aims to investigate the influence of anions on the self-assembly morphology and shape using a synthetic peptide of FFFFKK. Circular Dichoism (CD) and Environmental Scanning Electron Microscope (ESEM) were used to determine the secondary structure and self-assembly morphology, while Image J imaging software was used to measure diameter size. In the absence of anion, FFFFKK formed anti-parallel $\beta$-sheet that adopted sizeable fibrillar structure with a minimal increment over the first 7 hours of assembly. Irregular structure was observed in the presence of Iodide ion (I$^-$) with a less stable secondary structure such as $\beta$-turn and $\beta$-loop. In the presence of perchlorate ion (ClO$_4^-$), needle-like structure was observed with predominantly $\beta$-sheet structure. Our study showed that peptide morphology can be controlled by using different anions with careful selection of amino acid residues in peptide sequence.

1. Introduction
Molecular self-assembling is the association of molecular building blocks that spontaneously organized into ordered macroscopic structure by non-covalent bond without external control [1, 2]. Over the years, the preference to study the behavioral growth of self-assembled peptide nanostructures (SPNs) has expanded [3, 4] due to its biocompatibility and chemical diversity. Many short amphiphilic peptides are commonly used as scaffolds to produce inorganic materials that are in one-, two- or three-dimensions at nano-scale [5, 6]. These peptides have also been successfully used to fabricate materials with well-defined size and shape that have applications in various industry such as drug delivery, wound healing and tissue engineering [7-9]. The assembly mechanism occurs within the existence of several factors including peptide-peptide interaction [2]. However, interruption of external stimuli such as anions and changes in pH or temperature will cost the interaction of either forming a functional or non-functional structure [2]. These self-assembling structures are relatively discrete and unstable. Therefore, the ability to control the rational design peptides into targeted structures at the early stages of self-assembly should be further developed. This study provided insights into the role of anions on the structure formation over the first seven hours of the assembly. We designed a synthetic peptide, Ac-FFFKK-CONH$_2$ that consist of four hydrophobic amino acids, phenylalanine, (F) and two positively charge hydrophilic amino acids, lysine, (K) at pH 7. The study was carried out at room temperature in solutions with (I$^-$ or ClO$_4^-$) or without anions. The assembly growth monitoring and characterisation were performed at the intervals of three to four hours. This study provided insights to enhance our mechanistic understanding of the peptide self-assembly and offer the strategy of controlling the assembly growth.
2. Materials and methods

2.1 Materials
Sodium iodide and sodium perchlorate were purchased from Sigma-Aldrich. FFFFKK was synthesized based on the standard Fmoc solid-phase synthesis strategy [1] and purified by reverse-phase high-performance liquid chromatography indicating the high purity of at least 98%. F is phenylalanine representing a neutral charged hydrophobic amino acid and K is lysine, a positively charged hydrophilic amino acid.

2.2 Sample Preparation
Purified peptide was dissolved in solutions containing either distilled water or anions (130 mM of I\(^-\) or ClO\(_4\)\(^-\)) to create solutions at a final concentration of 1.0 mM, that is above the critical self-assembling concentration (CSAC) of both the peptides. Concentration of salt solutions were 130 mM, similar to the standard salt concentration in human blood that is between 135 – 145 mM [10]. Solutions were stirred continuously resulting homogenous solutions before further characterization.

2.2.1 Environmental Scanning Electron Microscope (E-SEM)
Samples for E-SEM were recorded on a JEOL JSM-6701F electron microscope. The magnifications used to observe the growth were varied according to the practicalities on observing the samples from as low as 130x to 40000x. A small volume (10 μL) of fresh or aged peptide solution was dropped onto an aluminum tape glued on top of the SEM sample stub. Once absorb the sample were copper coated by a sputter coater. Then, the samples were placed on the stage of the E-SEM and inserted into the vacuum chamber to be viewed.

2.2.2 Circular Dichroism (CD)
The CD measurements were performed on a JASCO’s J-1500 spectrometer using a 1 mm path length quartz cuvette. The CD spectra were recorded at room temperature with wavelengths ranging from 180 to 260 nm and a scan speed of 3s interval on average. The bandwidth was set to 2 nm [11] and an integrated Hg lamp was used as the light source. Before the reading of the sample was taken, the solvent background was subtracted and the spectra could be smoothed [11]. The resultant CD signals were expressed as mean residue ellipticity, [θ] which is in deg. cm\(^2\). dmol\(^{-1}\) unit versus wavelength.

2.2.3 Digital Image Analysis
Diameter of the structure was measured with the aid of Image J 1.43 (NIH) imaging software using eSEM images. The analysis was carried out by setting the measure scale to suit with the 2D eSEM image. Other detail and required measurements were set up in this step. Next, the boundaries of each structure on the image were detected. The boundary can easily be detected from binary images or other types, which were obtained from the threshold SEM images. Measurements were performed on regular structures only.

3. Results and discussion
Critical self-assembly concentration (CSAC) of FFFFKK was determined prior to the morphology study and secondary structure analysis. An analogy to CSAC has been drawn to the critical micelle concentration (CMC) of amphiphilic molecules such as surfactants. An amphiphilic peptide may display similar concentration-dependent self-assembly behavior, leading to the formation of peptide nano-structures [12]. We performed conductivity measurement to determine the CSAC values of FFFFKK as a function of peptide concentration. A series of peptide solutions with concentrations ranging from 0.02 to 2.0 mM was prepared by diluting the stock solution with distilled water. The conductivity of the peptide solution at different concentration was recorded by a conductivity meter. Below the CSAC, the addition of peptide molecules to the solution causes an increase in the number of charge carriers and consequently, an increase in the conductivity. Above the CSAC, further addition of peptide molecules increases the micelle concentration while the monomer concentration remains approximately constant. Since micelle is much larger than a peptide monomer, it diffuses more slowly
through the solution and is a less efficient charge carrier. A break on the plot of conductivity against peptide concentration is the CSAC. The plotted results suggested that FFFFKK has CSAC value of 0.25 mM (data not shown). In such case, peptides were able to self-associate since all the work was performed at a concentration of 1.0 mM.

**Table 1.** Average diameter of the structure formed in peptide solutions containing distilled water, iodide ion or perchlorate ion.

| Time/hour | Water | Iodide ion | Perchlorate ion |
|-----------|-------|------------|----------------|
| 3         | 2.8   | Irregular structure | 1.1 |
| 7         | 3.2   | Irregular structure | 1.4 |

**Figure 1.** The ESEM images of FFFFKK after 3 hours of growth in: (a) Water, (b) iodide ion, (c) perchlorate ion; after 7 hours of growth in: (d) water, (e) iodide ion (f) perchlorate ion.

SEM images were measured to examine the self-assembly structures visually in the samples at different time intervals. Significant changes in the morphology and size were observed when FFFFKK dissolved in different aqueous milieu, either in the form of fibril, needle-like or amorphous structure as shown in Figure 1. In Table 1 is shown the average diameter of the structure formed when FFFFKK is dissolved in different solutions. FFFFKK formed fibrillar structure in water with diameter of 2.8 µm in the first three hours. It grew to 3.2 µm after 7 hours in the same solution with very low density. FFFFKK formed needle-like structure in solution containing ClO₄⁻ with lower diameter between 1.1 to 1.4 µm. Screening effect from anion to overcome the electrostatic is prominent in this case in which higher density of needle-like structure was observed. The formation of needle-like structure is most likely due to the higher tendency of ClO₄⁻ to induce micelisation [16] than I⁻. Irregular and porous structure was found in the presence of iodide ion indicating the structure was not stable. Diameter measurement was not able to perform due to the limitation of the Image J imaging software.

Phenylalanine (F) is a strong hydrophobic amino acid that has a high tendency in forming β-sheet structure due to its hydrophobic effect [15]. CD results depicted in Figure 2 suggest that different packing geometries of β-sheets, β-loop and β-turn were present during the peptide assembly. In the distilled water, assembly structure remains to be anti-parallel β-sheet for 7 hours with proximate minimum at 195 and 220 nm respectively. In solution containing iodide ion, β-loop structure was observed within the first three hours with an approximate negative minimum at 200 and 220 nm. The structure was later evolved to β-turn with weak signal at 195 and 210 nm. In the presence of
perchlorate ion, FFFFKK formed needle-like structure with weak $\beta$-sheet signal at 195 and 215 nm initially. A strong $\beta$-sheet signal with negative minimum at 215 nm was observed with higher density of needle-like structure at the later hours.

![Figure 2. The CD spectra of FFFFKK during the first 7 hours in a) distilled water, b) sodium iodide and c) sodium perchlorate.](image)

Phenylalanine has the ability to form a stable hydrogel despite its aromatic residue [16]. Subsequently, the observation of different supramolecular architecture with the presence of anions indicated that the protonated lysine residues were neutralized. Drastic changes in morphology and shape when FFFFKK was in solutions containing $\Gamma$ or $\text{ClO}_4^-$ was most likely attributed to the complex interplay between neutralized electrostatic repulsion of charged lysine side chains and attraction energy between the structures [18]. The anions ($\Gamma$ and $\text{ClO}_4^-$) were effective in screening the charge-charge interaction between the peptide monomers through anion binding [19, 20]. Anions were able to penetrate closer to the hydrophobic region of the peptide in which the water/hydrophobic interface of the peptide enhances the adsorption of large and polarizable ions such as $\Gamma$ and $\text{ClO}_4^-$ [21]. Observation of less stable structures such as $\beta$-loop and $\beta$-turn in the presence of $\Gamma$ indicating that the self-assembly structures were prone to structural changes due to weak hydrogen bond and unstable NH-O angle [21]. The presence of needle-like structure in solutions containing $\text{ClO}_4^-$ is most likely due to higher micelle affinity of $\text{ClO}_4^-$, which favors the formation of $\beta$-sheet structure that eventually forms needle-like structure [14].

4. Conclusion
In this study, we have demonstrated the influence of anions on FFFFKK have the potential in producing different higher order self-assembled architectures in solution containing perchlorate ion. These structural changes have been well-correlated with the interplay of the non-covalent forces including hydrophobic interaction and electrostatic repulsion. Formation of $\beta$-sheet structure drives the formation and growth of fibrillar structure. It is noticeable that the presence of anions ($\Gamma$ and $\text{ClO}_4^-$) has a remarkable effect on the morphology. FFFFKK formed irregular structures in solution containing $\Gamma$. The peptide formed needle-like structures in large amount with the presence of perchlorate ion. Relatively less stable structures such as $\beta$-turn and $\beta$-loop was found in the early hours of assembly. With different preferences in promoting secondary structures, systematic changes in peptide sequences and charge distribution enable us to study the linkage between molecular architecture and self-assembled structure. Further study will be carried out to monitor the morphological changes of the peptide, including others in the series (FFKKFF and KFFFFK) at longer hours (up to 48 hours). This study provides insights to the future design of self-assembled nanomaterials in a rational way, such as scaffolds for tissue engineering and bone implant.

Acknowledgments
The authors would like to thank the Faculty of Chemical Engineering of Universiti Teknologi MARA for the financial support.

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