

Tunable Fmoc-Phe-(4-X)-Phe-NH₂ nanostructures by variable electronic substitution

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Supramolecular structures were produced by *in situ* enzymatic condensation of Fmoc-Phe-(4-X), where X denotes electron withdrawing or donating groups, with Phe-NH₂. The relative contribution of π -stacking and H-bonding interactions can be regulated by the nature of X, resulting in tuneable nanoscale morphologies.

Supramolecular self-assembly provides a route to fabrication of nanomaterials potentially useful in both high-tech and every-day life applications.¹⁻⁴ Peptide-based materials are of particular interest due to chemical diversity, biocompatibility and ease of synthesis.⁵ In particular, aromatic peptide amphiphiles, short peptide appended with aromatic ligands, are of interest due to its ability to self-assemble to a remarkable range of nanostructures with minimal molecular complexity.⁶⁻⁸

Biocatalysis is increasingly used for the *in situ* regulation of materials properties.⁹⁻¹² Proteases (and more recently lipases)¹³ provide a route for *in situ* formation of peptide nanostructures from amino acid precursors, by fully reversible condensation reactions¹⁴ which are driven by the energy contributions of molecular self-assembly, thereby overcoming the preference for hydrolysis rather than condensation in aqueous media. This approach avoids the formation of kinetic aggregates in favour of thermodynamically preferred structures thus allowing for reproducible self-assembly of hydrophobic peptide derivatives.¹⁵ Because these reactions operate under thermodynamic control, the approach allows for direct correlation of condensation yield with thermodynamic stability of peptide structure formed, as previously shown in dynamic combinatorial libraries.¹⁶

It is clear that there are options for controlling the morphology of self-assembling peptide nanostructures. There are two main approaches to achieve control over morphology, either by changing the self-assembly pathway (kinetic control^{17,18}) or by changing the peptide sequence.¹⁹ The self-assembly of these structures is dictated both by H-bonding interactions and aromatic stacking contributions²⁰⁻²² and it appears that the relative contributions of each of these can control nanoscale morphology. Indeed, systematic variation of the side chain of amino acids has a remarkable effect on the formed structures (spheres, tubes, fibres, tapes and sheets).²³

Nilsson's group recognised that the relative contributions of aromatic stacking interactions may be manipulated by using substitutions on aromatic (Phe) side chains. Indeed, they demonstrated that atomic substitution on amino acids side chains have a significant effect on self-assembly and the subsequent hydrogelation.^{24, 25} Gazit and Reches studied the effect of diphenylalanine modification on self-assembled nanostructures diluted from organic solvents.²⁶ Changing the electronic properties of benzyl group of Fmoc protected phenylalanine (Fmoc-Phe) with different halogens (F, Cl, Br) and at different positions (*ortho*, *meta*, *para*) could control the rate of self-assembly and mechanical properties.²⁵ Electronic modification on phenylalanine containing

peptides has also been used to study peptide/carbon nanotubes interactions²⁷ and to investigate the effect of aromatic interactions on the formation of amyloid-like structures.²⁸

In this work, we study electronic modification *via* different substituents and combine this with enzymatic self-assembly under thermodynamic control to allow for direct comparison of structures formed. Specifically, we investigate (i) the effect of electronic substitution on the interplay of aromatic stacking and H-bonding (ii) the ability to exploit effects of electronic substitution for morphological control of supramolecular nanostructures.



Scheme 1. Thermolysin catalysed amide bond formation of Fmoc-dipeptide derivatives containing groups with different electronic density.

We studied the supramolecular assembly of an amidated dipeptide aromatic derivative, **Fmoc-Phe-(4-X)-Phe-NH₂** produced by thermolysin catalysed amide condensation (**Scheme 1**). X represents a range of substituents on the *para* position of the first amino acid (OH, OPO₃H₂, H, F, CN or NO₂). Substituents were chosen on the basis of Hammett- σ_p values,²⁹ a measure of electronegativity (see **Table S1** ESI). In order to drive amide bond formation we used 20 mM of the modified amino acid **Fmoc-Phe-(4X)-OH** and 4 times excess (80mM) of the nucleophile **H-Phe-NH₂** in the presence of 1 mg/ml thermolysin (from *Bacillus thermoproteolyticus Rokko*) in 100 mM sodium phosphate buffer at pH 8 based on the previously reported procedure.¹⁶ For all samples the final pH value was found to be 8 during the enzymatic condensation/self-assembly process except from the phosphorylated derivative (2) which found to be 7.6. Amide bond formation expressed as percentage of conversion was determined by reverse-phase high performance liquid chromatography (HPLC), **Figure 1a**. Dipeptide derivatives show variable yield conversion after 24 hours of enzyme addition as summarized in **Table 1**. Compared with the un-substituted Fmoc-Phe-Phe-NH₂ (82% conversion), higher conversions were observed when using electron donating groups (OH, 100%) followed by the phosphorylated derivative (OPO₃H₂, 87%), both reaching their final conversions within 30 minutes. When using electron withdrawing groups, lower conversion yields were observed (59-77%). Therefore, there is a clear trend between the electron density on the benzyl group and the conversion yields, implying that aromatic interactions provide an important driving force for self-assembly. It should be noted that there is also a correlation with morphological change, with the electron donating substituents giving rise to suspensions and electronic withdrawing groups giving gels.

Table 1. Summary of %Conversion values after 48 hours of enzyme addition and the corresponding appearance change.

Entry	Precursor	%Con.	Transition (if any)
1	Fmoc-Tyr	100	gel to suspension
2	Fmoc-Phe-(4-OPO ₃ H ₂)	87	solution to suspension
3	Fmoc-Phe	82	suspension to gel
4	Fmoc-Phe-(4-F)	59	suspension to weak gel
5	Fmoc-Phe-(4-CN)	77	transparent gel to translucent gel
6	Fmoc-Phe-(4-NO ₂)	60	translucent gel (no change)

In order to acquire insights into the changing supramolecular interactions, we used fluorescence spectroscopy to measure changes in fluorenyl group environment.^{30, 31} Some of the materials studied here are highly scattering and care should therefore be taken with interpretation of the results. With this in mind, we focus on spectral shape (changes in emission wavelength, **Figure 1b**) rather than intensity.

The fluorescence emission of the precursors **1** and **2**, exhibit a similar main peak at 319 nm and an excimer peak at 360 nm (full spectra shown in **Figure S1**) (clearer for **1**) which was previously seen for Fmoc-amino acid precursors which form micellar aggregates.^{32, 33} After the addition of thermolysin and upon condensation and assembly, 8 nm red shift was observed for **1** and 14 nm for **2** for the main peak while the excimer disappeared, these observations suggested supramolecular reorganisation³⁰ which was further investigated below. For **3** the main emission peak was detected at 322 nm. In this case, *in situ* condensation by thermolysin and consequent self-assembly induces a red-shift (330 nm) and an excimer peak appears covering the region from 355-380 nm, accompanied by a substantial decrease in the relative emission due to the formation of extended stacking interactions among the aromatics (**Figure S1**). The fluorinated phenylalanine derivative **4** shows an emission peak at 327 nm. Condensation reaction led to a small red-shift (329 nm) with the emission peak significantly quenched. Substantial quenching in the main peak emission was also observed for **5**. Biocatalytic assembly of **6** did not show significant differences (1 nm) in the fluorescence spectrum indicating little change in the fluorophores arrangement. The substitutions impact on the aggregation behaviour of the precursors, with critical aggregation concentration (CAC) values dictated by the characteristics of the substituent was investigated (**Figure S3**). Overall, the trends suggest that introduction of a donor (OH) or bulky phosphorylated group (OPO₃H₂) give rise to the most substantial red-shifts, suggesting more optimised stacking interactions among the aromatics. As the electron density of the benzyl group reduced *via* the electron withdrawing groups a much smaller red-shift structure was observed while no difference was noticed for the strong electron withdrawing group (NO₂). These results indicate that for substituents with electron withdrawing groups hydrophobic contributions play a less important role on the molecular self-assembly.

Next, we investigated the propensity of the substituted dipeptide derivatives to form hydrogen bonding arrangements *via* the amide groups of the backbone using FTIR spectroscopy, **Figure 1c**. For **1** and **2**, weak peaks observed in the region of 1625-1638 cm⁻¹ and at 1684 cm⁻¹ correspond to formation of β -sheet-like hydrogen bonding of the amide and stacked carbamate groups, respectively.²⁰ As the π -cloud of the benzyl group was reduced by the acceptor groups, stronger β -sheet-like hydrogen bonding was identified. As no evidence for interactions with the substituent groups was found, this effect is attributed to a difference in the distribution of electron density in the molecule. These observations suggest an inverse correlation between the contributions of H-bonding and aromatic

stacking, which is dictated by the electron density the aromatic side chain.

Having established the relative importance between hydrophobic interactions and hydrogen bonding, we used Circular Dichroism (CD) spectroscopy to investigate the effect on supramolecular chirality resulting from the altered balance between aromatic stacking and H-bonding (**Figure 1d**). For **1** and **2**, a strong positive CD signal for Fmoc group (307 nm) was detected. The CD signal was reduced when peptides were modified with less electron density groups (**3, 4**). Introduction of electron withdrawing groups (**5, 6**) shows opposite behaviour, giving negative signal for Fmoc group (303 nm and 310 nm respectively).

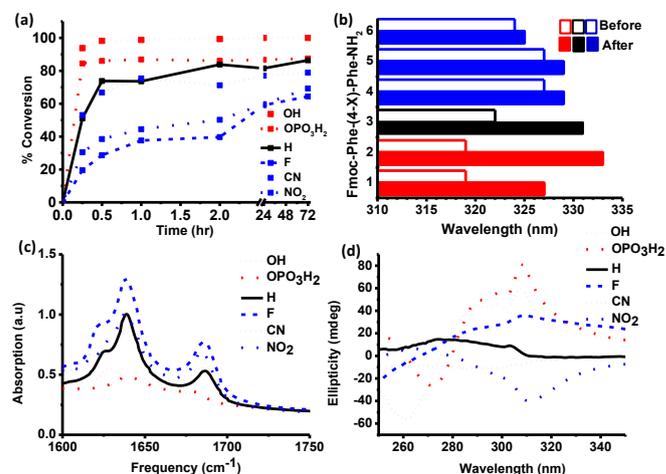


Figure 1. HPLC data, illustrating the percentage of peptide conversion of different diphenylalanine derivatives over time ($\lambda=280$) (a) Histogram illustrating the difference of wavelength shifting before and after the *in situ* condensation reaction and assembly (b) FTIR amide region of diphenylalanine derivatives in D₂O (c) and CD spectra of the diphenylalanine derivatives (d) (for corresponding high tension (HT) voltage see **Figure S2**, **ESI**).

Regarding the effect of electronic modification on hydrophobic contributions and hydrogen bonding interactions, we can conclude that the rich electron density group (OH) as well as the bulky phosphate group (OPO₃H₂) give rise to better stacking interactions among the aromatics with less hydrogen bonding capacity. An opposite observation was revealed by the introduction of electron withdrawing groups. From the above observations we can conclude that substitution with electron donating or withdrawing groups can enhance or disrupt the delicate balance between aromatic interactions and hydrogen bonding which drive the molecular self-assembly.

Nanoscale morphology was then observed using transition electron microscopy (TEM). Remarkably, different supramolecular architectures were obtained by altering the electron density of the phenylalanine residue. The base material, Fmoc-Phe-Phe-NH₂ self-assembled into a fibrillar network similar to that observed for Fmoc-Phe-Phe.^{34, 35} Upon substitution with OH, sheet-like structures were obtained³⁶ while tube-like morphology was seen for the OPO₃H₂ derivative, possibly due to the bulky nature of the side chain which cannot be incorporated in a two dimensional sheet. The introduction of strong electron withdrawing groups (CN, NO₂) did not lead to

change the nanoscale morphology. These observations are in line with spectroscopy results.

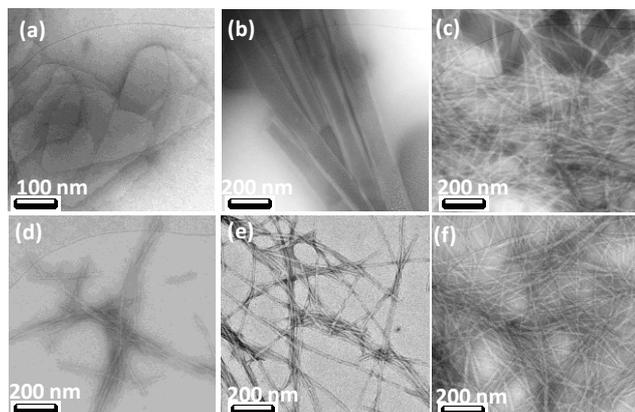


Figure 2: TEM images of aromatic diphenylalanine derivatives after 48 hours of thermolysin addition. (a-f corresponds to **1-6**, respectively).

In conclusion, we have demonstrated that different supramolecular structures can be produced by changing the electronic properties of phenylalanine on **Fmoc-Phe-(4-X)-Phe-NH₂**, where X is electron donating or withdrawing group (OH, OPO₃H₂, F, CN, NO₂). Fibrillar nanostructures can be obtained in case of withdrawing groups and the neutral phenylalanine (H) but sheet-like structure for the tyrosine derivative and tubular nanostructure for the phosphorylated dipeptide. The electronic change of the phenylalanine amino acid residue gave rise to tune the stacking interactions among the aromatics and the hydrogen bonding between the dipeptides which might be useful in developing biocompatible electronics and sensing technology. We believe that the interplay between stacking interactions and hydrogen bonding which can be enhanced or disfavoured by different electronic modifications may give rise to different nanoscale architectures.

Notes and references

The research leading to these results has received funding from the European Research Council under the European Union's Seventh Framework Programme (FP7/2007-2013)/EMERgE/ERC Grant Agreement No. (258775). CGP would like to thank Linn Products Ltd for funding. YMA acknowledges FP7 Marie Curie Actions, via the initial training network ReAd (Contract No. 289723) for funding. We also acknowledge Margaret Mullin from University of Glasgow for help in TEM imaging.

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