Formation of Mixed Ionic Complementary Peptide Fibrils

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Summary: Two peptides (FEFEFKFK and HHHHHHFEFEFKFK) have been synthesised and their phase diagrams mapped out as a function of concentration and temperature. Both peptides formed self-supporting fibrillar hydrogels above a similar critical molar gelation concentration with a fibril diameter corresponding to the length of the fully stretched monomer. Mixing the peptides in a 9:1 ratio of FEFEFKFK to histadine functionalized FEFEFKFK in the presence of nanogold particles (binds to the histadine groups) resulted in thicker fibres suggesting that two fibrils associate together to form a fibre. TEM studies revealed that the gold particles were distributed throughout the hydrogel and adjacent to both sides of the fibrillar structures with an average distance between particles of 21 nm. It is postulated that the peptides form anti-parallel beta sheet fibrils that associate together via π-stacking interactions between the imidazole side chains of the histadine groups to form a fibre, where on average 1 in every 44 peptides is functionalized.

Keywords: electron microscopy; hydrogel; peptide; self-assembly

Introduction

Molecular self-assembly is a powerful tool for the fabrication of supramolecular structures and advanced materials with a wide variety of properties. The inspiration for the development of such structures often comes from the complex self-assembling behavior of proteins and polysaccharides encountered in Nature. Typically the complex structures formed from natural systems are all derived from simple building blocks such as amino acids, nucleic acids and lipids. Peptide based molecules have recently attracted considerable attention for the production of ordered nanostructures, where many studies have demonstrated their ability to form well organized assemblies through non-covalent interactions such as hydrogen bonds, electrostatic interactions, van der Waals forces, π-stacking and hydrophobic interactions.[1–4] These include cyclic peptides designed with alternating D- and L- amino acids,[5] amphiphile peptides,[6] peptide-conjugates[7] and ionic self-complementary peptides.[8] Many nanostructures formed by these self-assembling peptides are promising for applications in various fields including molecular electronics, tissue engineering, drug delivery and material science.

One class of self-assembling peptides are 'ionic complementary' peptides, first discovered by Zhang in 1992.[9] They have a structure of alternating hydrophobic and hydrophilic amino acid residues which assemble via hydrogen-bonding of the backbone of the peptide to form β-sheet structures in solution. These fibrils have a hydrophobic face on one side of the peptide, and a hydrophilic face on the other. The hydrophilic amino acids are composed of an equal number of positively and negatively charged residues, which give rise to electrostatic interactions. It is known that increased hydrophobicity can help drive

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this process,[10] while the burying of hydrophobic faces and electrostatic interactions are thought to be a factor in the formation of higher order fibrils and fibers.[11,12] Many studies have shown that by varying the amino acid residues used, charge distribution, length of the peptide chain and concentration, control over assembly can be achieved.[4,10,12] More recent attention has focused on incorporating bioactive peptides into fibrillar structures to tailor properties and create fibrillar networks with enhanced properties for a specific end use. One example of this is the direct coupling of specific cell adhesion motifs (including RGD, DGR, ALK) to the well known self assembling peptide RADARADARADARADA (RAD16-I) (where R is arginine, A is alanine and D is aspartic acid), and mixing bioactive peptide with the pure RAD16-I peptide to enhance cell migration and growth within a 3-dimensional fibrillar peptide scaffold.[13] Subsequent cell culture studies on such mixed systems exhibited both enhanced cell migration and growth. Little is known however if, or how, the funtionalised peptide has distributed itself throughout the network structure.

Here we aim to examine the distribution of a functionalized peptide into the fibrillar structure of the well known β-sheet assembling peptide FEFEFKFK, where F is phenylalanine, E is glutamic acid and K is lysine.[14] A histidine (H) functionliased peptide HHHHHHFEFEFKFK was selected as the functionalized peptide as the FEFEFKFK peptide will allow the peptide to incorporate itself within the pure peptide fibril while the H groups allow covalent bonding of the peptide to nickel nitrilotriacetic acid (Ni-NTA) nanogold by binding to the nickel nitrilotriacetic acid functionality (see Figure 1), while. The binding process of the nanogold requires only two histidine amino acids, as shown in Figure 1, however we anticipate the binding process to be more efficient when using a sequence of six H amino acids. The nanogold particles can then be viewed in the TEM, allowing determination of the position of histidine peptide.

Materials and Methods

Materials and Peptide Synthesis

Amino acids, activator (HCTU) and Wang resin were purchased from Novabiochem (Merck) and used as received. All other reagents and solvents were purchased from Aldrich and used without further purification. FEFEFKFK and HHHHHHFEFEFKFK were both synthesised *via* standard solid-phase synthesis[15] on a ChemTech ACT 90 peptide synthesiser (Advance ChemTech Ltd., Cambridgeshire, UK), using Fmoc-Lys(Boc)-Wang resin (mesh = 200, loading = 0.7 mmol g^{-1}); coupling of amino acids was done using standard solid phase synthesis protocols with HCTU as activator; deprotection of

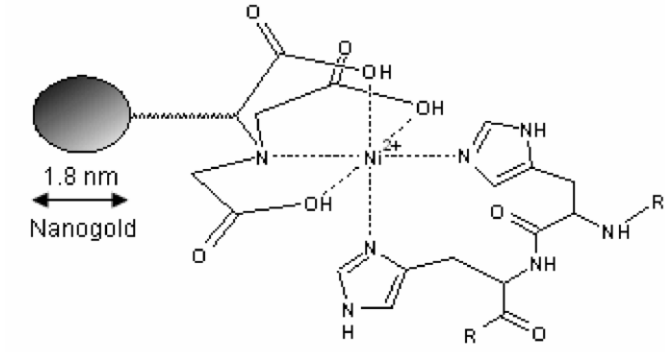

Figure 1.
Interaction of two histidine amino acids with Ni-NTA-nanogold.

side groups was done using piperidine; cleavage was done by using a TFA-anisole mixture. Each reaction step was confirmed by the Kaiser test.[16] The peptide was recovered and washed in cold ether and freeze-dried. This last step was repeated 3 times. Both functionalized and un-functionalized peptides were characterised by ^{1}H NMR, HPLC and MALDI-tof mass spectrometry and their purity estimated to be $\geq$90% and $\geq$95% respectfully. The nanogold for the histidine tagging experiments was purchased from nanoprobes at a concentration of 10 nanomols ml^{-1}.

Solutions and Gel Preparation

Gels and solutions were prepared directly in Hellma quartz cells with an optical path length of 5 mm by dissolving the desired amount of peptide in water at 90 °C. The solutions were agitated at high temperature for 10 to 20 minutes depending on the concentration and peptide used to ensure full dissolution. All the solutions had a pH in between 6 and 7. The solutions were then cooled down at room temperature. Depending on the concentration and peptide used gelation occurred during cooling. Samples containing nanogold were prepared in a similar way; dissolving the FEFEFKFK and HHHHHHFEFEFKFK peptide in doubly distilled water in a 9:1 ratio by weight, adding the nanogold in a ratio of nanogold to HHHHHHFEFEFKFK of 10:1 by weight, incubating the sample at 85 °C for 24 hours before cooling to room temperature.

Transmission Electron Microscopy (TEM)

Carbon-coated copper grids (400 mesh, Agar scientific) were glow discharged for 5 seconds and placed shiny side down on the surface of a 10 μl droplet of sample for 10 seconds. Typically, samples were prepared by diluting 10 fold in water and agitating vigorously to separate fibrillar structures. Loaded grids were immediately placed on a 10 μl droplet of double deionised water for 10 seconds and subsequently blotted. Washed grids were then placed on a 10 μl droplet of freshly prepared and filtered uranyl acetate solution (4% w/v) for 60 seconds for negative staining and then blotted continuously against double folded Whatman 50 filter paper. Data were recorded in a Tecnai 10 TEM operating at 100 keV (calibrated magnification of 43,600×) onto Kodak SO-163 film. Images were subsequently scanned using a UMAX2000 transmission scanner providing a specimen level increment of 0.366 nm pixel^{-1}.

Results and Discussion

Phase Diagrams

The gelation behvaiour of both FEFEFKFK and HHHHHHFEFEFKFK were studied studied initially using the standard tilt test method and their phase diagrams are given in Figure 2.

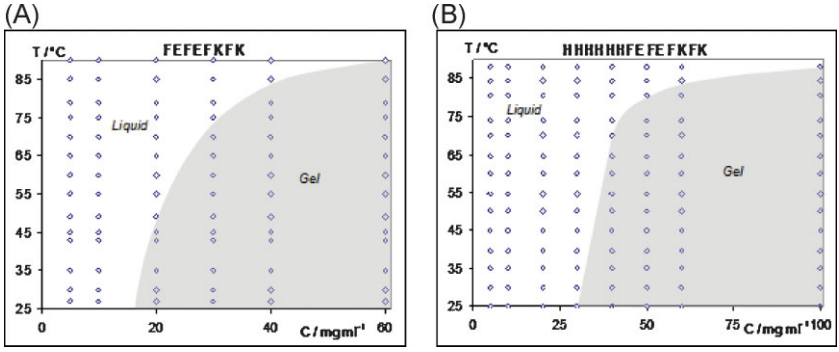

Figure 2.
Phase Diagrams for (A) FEFEFKFK and (B) HHHHHHFEFEFKFK.

It can be seen that the addition of histidine amino acids onto the FEFEFKFK structure has increased the critical gelation concentration of peptide from 17 to 30 mg ml^{-1}. These concentrations correspond to the molar concentrations of 0.0154 M and 0.0152 M respectively, showing that a similar number of molecules are required to assemble into fibrils and subsequently into a self-supporting hydrogel. The liquid-gel phase boundary with respect to the temperature is slightly steeper for the functionalized peptide in comparison to the unfunctionalized one, otherwise they are simlar. Again, similar molar concentrations of peptides (~0.0514 and 0.0535 M for the functionalized and unfunctionalized peptide respectively) are required to undergo the transition from gel to liquid at 90 °C. This shows that the gelation behaviour of the peptides is very similar.

Transmission Electron Microscopy

Transmission electron microscopy (TEM) was used initially to study the fibre structure of pure FEFEFKFK and HHHHHFEFEFKFK peptides (Figures 3 and 4 respectively). It is evident from Figure 3 that FEFEFKFK peptide forms a network of branched fibrils at 5 mg ml^{-1} where fibril diameter is $\sim 2.9 \pm 0.2$ nm, matching the width of one fully stretched peptide monomer, while fibril length is several microns. These dimensions match those we extracted previously from small angle neutron scattering experiments.[14]

Figure 4 shows micrographs obtained for HHHHHHFEFEFKFK over a range of concentrations (5–30 mg ml^{-1}). Fibrils that are several microns in length with a diameter of 4.2 ± 0.5 nm were visible at all concentrons, which is in reasonable agreement with the theoretical length of the fully stretched peptide (~5 nm). At 5 mg ml^{-1} (Figure 4A) a low density of fibrils is observed, however, fibrils appear to be lying flat on top of each other which is most likely due to the drying and collapse of the sample during grid preparation. At the same concentration FEFEFKFK (Figure 3) showed a greater density of fibrils in comparison to its H functionliased equivalent. This is due to a greater number of unfunctionalized FEFEFKFK molecules being available and contributing to fibril formation. It is also interesting to note that the HHHHHHFEEFEFKFK fibrils appear more flexible that those formed from FEFEFKFK. As the concentration of HHHHHHFEFEFKFK increased the denisty of fibrils began to increase and fibril entanglements were observed (see rectangular highlighted areas in Figure 4) which are likely to be responsible for the macroscopic gelation of the sample (Figure 4D).

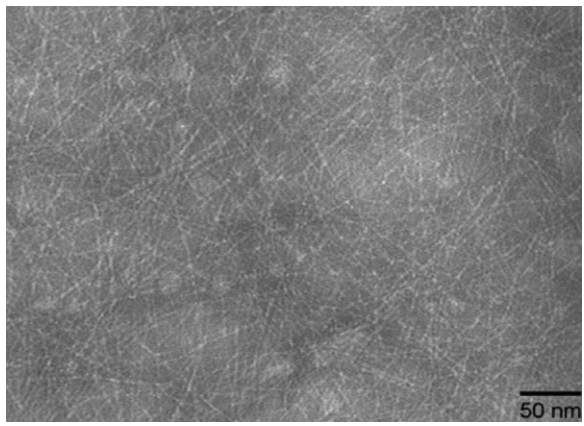

Figure 3.
TEM micrograph of FEFEFKFK at 5 mg ml^{-1}. The scale bar represents 50 nm.

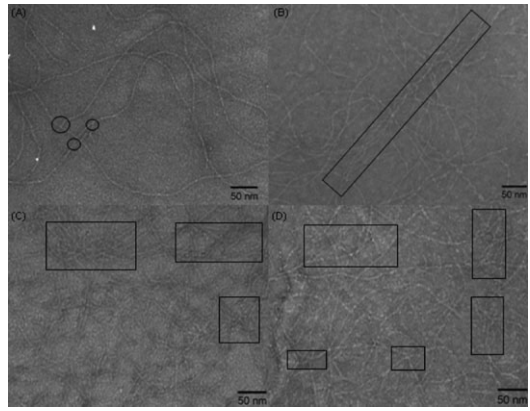

Figure 4.
TEM micrographs of HHHHHHFEFEFKFK with concentrations of (A) 5, (B) 10, (C) 20, and (D) 30 mg ml^{-1}. Circles indicate branch points, while rectangles indicate entanglements. In each case the scale bar represents 50 nm.

To examine the how the functionlised peptide mixes with its un-functionalized equivalent a 5 mg ml^{-1} peptide sample was prepared containing FEFEFKFK and HHHHHHFEFEFKFK in a 9:1 ratio with a 10:1 ratio of nanogold to functionliased peptide. The nanogold can covalently bind to the histadine of the functionalized peptide, hence the location of any gold particles adjacent to fibrils in the TEM micrographs would indicate the presence of a functionalized peptide within the fibrillar structure. Figure 5 shows two typical TEM micrographs obtained. Along the fibril structure it is possible to see black dots, such as those highlighted by the circles in Figure 5A. The size of these dots is 1.9 ± 0.3 nm, which corresponds to the size of the nanogold particles (1.8 nm). The presence of such gold particles adjacent to the fibrils indicates that HHHHHHFE-FEFKFK has indeed been incorporated throughout the fibrillar structure. It is also possible to see some twist in the fibrils, such as in the areas highlighted by rectangles in Figure 5A. The nature of the entanglements makes it difficult to judge the regularity of this twist however. The fibril width was determined to be $\sim 6.8 \pm 0.5$ nm which is greater than expected for a single fully extended HHHHHHFEFEFKFK ($\sim$5 nm). This indicates a change in the interactions and/or arrangement of the β-sheet structure, leading to fibrils of a larger diameter. The overall fibril width could conceivably correspond to the width of two FEFEFKFK molecules, which have an extended peptide length of 3 nm per molecule. As we have

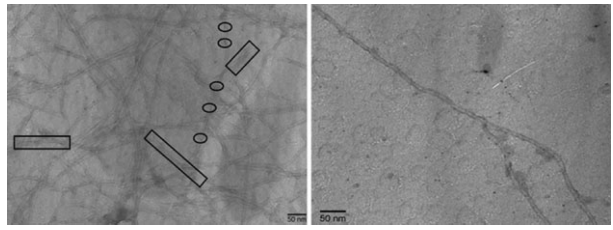

Figure 5.
TEM micrographs of a 9:1 peptide mixture of FEFEFKFK and HHHHHHFEFEFKFK at a concentration of 5 mg ml^{-1} diluted in 250 μl of nanogold for 24 hrs at 85 °C. Circles highlight gold particles, rectangles highlight twists in the fiber structure. The scale bar represents 50 nm.

shown that FEFEFKFK forms fibrils with a width of 2.9 nm, however, the addition of HHHHHHFEFEFKFK is likely to be the cause of the increased fibril width observed here. When HHHHHHFEFEFKFK is incorporated within a fibril, it is likely that HHHHHH will stick out from the edge of the fibril. If two of these HHHHHH groups on different fibrils interact then this could lead to the association of two fibrils. The width of this new fibre would be ~8 nm assuming the peptides were fully stretched, and corresponds to a diameter of 7.1 nm predicted from our experimental values (4.2 + 2.9 nm). This diameter is in reasonable agreement to the fiber width observed experimentally.

Figure 5B shows a branched fiber from the nanogold experiment. It is possible to see nanogold particles dotted along both the left and right side of the fiber indicating an anti-parallel β-sheet arrangement within the fibrillar structure.

Figure 6 shows the distribution of distances between gold particles that was observed in the fiber structure. The distribution of the particles range mainly from ~0–50 nm, with a peak in frequency at 10 nm indicating that the finctionalised peptide is distributed fairly evenly throughout the fibril and the network structure. Occasionally binding distances are much greater, with distances between gold particles reaching as high as 210 nm. Discounting the larger distances, the average distance between gold particles was determined to be 21 nm.

It is known that the typical hydrogen bonding distance between β-strands running perpendicular to the fibril axis in a peptide β-sheet is 4.7 Å.[17–19] Assuming this distance holds for our peptide system, and taking the average distance between functionalized peptides as 21 nm, then an average of approximately 1 in every 44 peptides incorporated into the fibrillar structure would correspond to a functionalized peptide. This should correspond to ~2.3% of the peptide monomers incorporated. We have, however, 10% by mass of HHHHHHFEFEFKFK, therefore, it would be expected that the histidine peptide would be ~6% of all peptide monomers within the fibril. Such a discrepancy between actual (2.3%) and predicted (6%) percentage of monomers incorporated is due to the fibril-fibril associations predicted from our TEM results. It should be remembered that the fibril width observed for the mixed peptide system

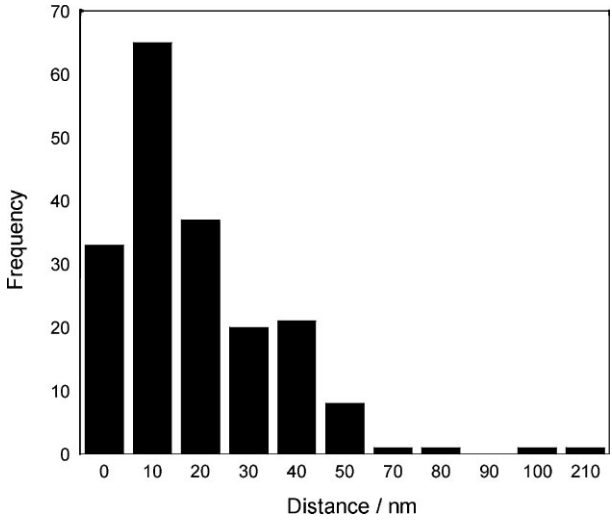

Figure 6.
Frequency of distance between gold particles observed in fibrils.

was greater than that for the pure HHHHHHFEFEFKFK fibrils, hence it was proposed that two fibrils came together to form one fibre, possibly driven by π-stacking interactions between the histidine imidazole side chains. Such fibril-fibril association would result in approximately half of the histidines being buried from the aqueous phase hence unable to bind with the nanogold, thus halving the number of free histadine binding sites. This model predicted is therefore in agreement with the number of free histadine groups observed experimentally.

Conclusion

Two ionic-complementary peptides, FEFEFKFK and HHHHHHFEFEFKFK, have been synthesised and found to form self-supporting hydrogels above a similar molar critical gelation concentration. Both peptides displayed similar phase behaviour with concentration and temperature, and both formed β-sheet rich fibrillar structures with a diameter corresponding to $\sim$1 fully stretched peptide monomer (2.9 ± 0.2 nm for FEFEFKFK, and 4.2 ± 0.5 nm for HHHHHHFEFEFKFK). The density of fibrils was found to be much greater for FEFEFKFK. When the peptides were mixed together in a 9:1 ratio of un-functionalized to histadine functionalized peptide in the presence of nanogold particles, thicker fibres formed that had a diameter of 6.8 ± 0.5 nm. Moreover gold particles were found to be distributed throughout and adjacent to both sides of the fibrillar structures with an average distance between particles of 21 nm. Such behaviour suggests that the peptides form fibrillar stuctures via anti-parallel beta sheets and that two fibrils associate together via the π-stacking interactions between the imidazole side chains of the histadine groups to form a fibre, where on average 1 in every 44 peptides is functionalized.

[1] S. Zhang, *Materials Today* **2003**, *6*, 20–27.
[2] R. V. Ulijn, A. M. Smith, *Chemical Society Reviews* **2008**, *37*, 664–675.
[3] I. W. Hamley, V. Castelletto, *Ange. Chemie Int. Ed.* **2007**, *46*, 4442–4455.
[4] P. Chen, *Colloids and Surfaces a-Physicochemical and Engineering Aspects* **2005**, *261*, 3–24.
[5] H. S. Kim, J. D. Hartgerink, M. R. Ghadiri, *JACS* **1998**, *120*, 4417–4424.
[6] S. Vauthey, S. Santoso, H. Gong, N. Watson, S. Zhang, *PNAS* **2002**, *99*, 5355–5560.
[7] F. Stoica, C. Alexander, N. Tirelli, A. F. Miller, A. Saiani Chem. Commun. **2008**, 4433–4435.
[8] Y. Hong, L. Lau, L. Legge, P. Chen, *Biomacromolecules* **2004**, *4*, 1433–1442.
[9] S. G. Zhang, C. Lockshin, et al. *Embo Journal* **1992**, *11*, 3787–3796.
[10] M. R. Caplan, E. M. Schwartzfarb, S. Zhang, R. D. Kamm, D. A. Lauffenburger, *Biomaterials* **2002**, *23*, 219–227.
[11] A. Aggeli, I. A. Nyrkova, M. Bell, R. Harding, L. Carrick, T. C. B. McLeish, A. N. Semenov, N. Boden, *PNAS* **2001**, *98*, 11857–11862.
[12] Y. Hong, L. Lau, L. Legge, P. Chen, *Journal of Adhesion* **2004**, *80*, 913–931.
[13] A. Horii, X. Wang, F. Gelain, S. Zhang, *PLoS ONE* **2007**, *2*, e190.
[14] A. Mohammed, A. F. Miller, A. Saiani, *Macromolecular Symposia* **2007**, *251*, 88–95.
[15] W. C. Chan, P. D. White, *Fmoc Solid Phase Synthesis*, Oxford University Press, New York, **2000**.
[16] E. Kaiser, R. L. Colescott, C. D. Bossinger, P. I. Cook, *Analytical Biochemistry* **1970**, *34*, 595–598.
[17] L. C. Serpell, P. E. Fraser, M. Sunde, *Methods in Enzymology* **1999**, *309*, 526–536.
[18] A. Aggeli, G. Fytas, D. Vlassopoulos, T. C. B. McLeish, P. J. Mawer, N. Boden, *Biomacromolecules* **2001**, *2*, 378–388.
[19] B. Ozbas, J. Kretsinger, K. Rajagopal, J. P. Schneider, D. J. Pochan, *Macromolecules* **2004**, *37*, 7331–7337.

