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Co-assembly of Fmoc-based self-assembling peptides results in the formation of homogenous nanofibres for high-density, concomitant peptide presentation.

Characterisation of Minimalist Co-Assembled Fluorenylmethyloxycarbonyl Self-Assembling Peptide Systems for Presentation of Multiple Bioactive Peptides

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Abstract

As novel biomaterials for tissue engineering, the hydrogels formed by self-assembling peptides (SAPs) enable the development of finely tuned cellular microenvironments suitable for a range of biomedical applications. Recently, the development of bioactive minimalist fluorenylmethyloxycarbonyl (Fmoc) peptide sequences has opened the possibility to provide both physical and biochemical cues for directed cell growth with minimal synthesis. However, due to a lack of understanding in the process and for ease of analysis, these systems typically consist of a single SAP, which results in chemically and biologically homogenous scaffolds. In order to improve the utility of these systems, significant opportunities lie in the controlled inclusion of several biological motifs via the co-assembly of multiple SAPs into a single structure. This potentially enables the concomitant presentation of multiple bioactive motifs in a single material, resulting in a sophisticated biomimetic scaffold. Here, we consider the incorporation of multiple Fmoc-SAPs into a single hydrogel scaffold through a) co-assembly and b) hydrogel-mixing. Two important extracellular matrix (ECM) protein-derived peptides: arginine-glycine-aspartate (RGD) and isoleucine-lysine-valine-alanine-valine (IKVAV) were included in minimalist Fmoc-SAPs and used to form co-assembled and hydrogel-mixed systems. Both systems were assessed in terms of their molecular arrangement, (nano)structure, and the mechanical properties of the hydrogel. Our results indicate that both approaches were successful, forming stable nanofibrous networks and hydrogels. Importantly, our results demonstrate that mixed gels formed a hydrogel consisting of a mixture of two homogeneous structures, whereas co-assembly facilitated a single randomly sorted nanofibrous assembly. This research demonstrates a facile method to functionalise this class of hydrogel, and provides a basis for future *in vitro* and *in vivo* studies of these systems for tissue engineering to assess the effects of the distribution and presentation of multiple signals.

Keywords

Self-assembling peptides; hydrogels; co-assembly; Fmoc; tissue engineering; IKVAV; RGD

The successful application of biomaterials in tissue engineering requires materials that have carefully tuned physical and chemical properties on biologically relevant scales to promote cellular regeneration while exhibiting little or no immune response.^[1, 2] In addition to being both biocompatible and non-cytotoxic after degradation, such materials must provide three-dimensional (3D) nanostructural support for cells as well as biochemical cues to promote and regulate cell behaviour such as cell adhesion, migration, proliferation and differentiation.^[1, 3, 4] These requirements present significant challenges in the design of tissue engineering materials, placing constraints on the types of materials that can be used and how their structures can be applied to cell regeneration and repair.

Nanostructured 3D matrix scaffolds are some of the most promising and important biomaterials that have been applied to tissue engineering.^[2, 5] Developing design rules for these materials allow them to be purpose-built to provide a suitable cellular microenvironment that mimics the native ECM.^[1-3, 6] A variety of scaffolds have been investigated including electrospun nanofibres,^[7-9] biologically derived and synthetic hydrogel scaffolds,^[10-16] and SAP hydrogels.^[2, 17, 18]

SAP hydrogel scaffolds have proven to be particularly promising due to their inherent biocompatibility, tuneable mechanical and chemical properties, ability to fill irregularly shaped voids, and their porous, nanofibrous structure, making them ideal candidates for applications in regenerative medicine.^[2, 19] They are a novel class of biomimetic materials containing a short sequence of amino acids (peptide).^[20, 21]

Recently, the potential of minimalist SAP systems for biomedical applications has gained much interest, due to their short length and ability to present biochemical cues at high densities.^[17, 19, 22] Underpinning the structural formation of one group of these systems, fluorenylmethyloxycarbonyl (Fmoc) SAPs, is π - β assembly.^[22, 23] Here, under appropriate conditions, the Fmoc groups stack on top of each other through π - π stacking forming the backbone of the Fmoc-SAP structure, and the pendant peptides align through hydrogen bonding to form a network of anti-parallel β -sheets.^[24] These assemblies subsequently close to form nanotubes with the peptide sequence exposed at high density on the surface.^[22] Individual nanotubes then align longitudinally, resulting in the formation of nanofibre bundles that constitute the hydrogel scaffold.^[19] The minimalist nature of Fmoc-SAP systems is advantageous over traditional SAP systems as they are relatively inexpensive and quick to produce.^[19, 23] In addition, the Fmoc moiety itself has been shown to possess anti-inflammatory properties, a valuable property for the development of adjuvant scaffolds in cellular applications.^[25]

Another promising advantage of Fmoc-based SAP systems over other, longer SAP systems is their heightened potential for biofunctionalisation. Previously, biofunctionalisation of non-Fmoc peptides (such as RADA16) and peptide amphiphiles (PA), achieved through capping with short bioactive sequences, has shown some promise in eliciting an improved cellular response *in vitro* and *in vivo*.^[17, 26-28] The capping of PAs with IKVAV in particular has seen selective promotion of cell differentiation, neurite extension, and neural differentiation, as well as reduced development of astrocytes (an inflammatory cell within the central nervous system (CNS)), indicating the formation of an appropriate cellular microenvironment for tissue engineering, in this case within the CNS.^[17, 29-31] While these results are promising, the relative size of the bioactive epitope to the PAs results in an inherently low-density presentation of the epitope.^[19, 31, 32] In addition, work on biofunctionalised PAs has shown

that density, rather than dimensionality, of the biofunctional epitope presentation plays a more important role in cell differentiation and is likely to play a wider role in the biofunctionality of the introduced material.^[26]

Our previous research has focused on the high-density presentation of RGD and IKVAV epitopes (Figure 1a & b).^[19, 33, 34] The laminin-derived IKVAV and the fibronectin-derived RGD sequences are common components of the mammalian ECM.^[19, 35] These Fmoc peptides have been rationally designed to form SAP hydrogels at physiological pH,^[19] allowing for control of various cellular processes including survival, adhesion, differentiation, migration and proliferation.^[2] Incorporation of these sequences has enabled us to develop an Fmoc-SAP scaffold that more closely mimics the ECM, both physically and chemically. However, while biofunctionalisation of SAP scaffolds represents a significant advancement in the development of cell scaffold materials, cellular control is somewhat limited in this instance by the presentation of a single biochemical cue.

Figure 1. (a) Structural formulae for the bioactive Fmoc-SAP sequences, (a) Fmoc-FRGDF, (b) Fmoc-DIKVAV with (a' & b') corresponding TEM images (scale = 200 nm). (c) π - β Co-assembly process where peptides are mixed prior to gelation and co-assembled via a pH switch method. (d) In contrast, the mixed assembly process results from the mixing of two previously prepared gels, giving rise to an inhomogeneous hydrogel.

The combination of multiple signals in a single, homogenous scaffold presents the potential for a synergistic effect on cellular response through the improved spatial and chemical arrangement of multiple bioactive signals.^[36] A range of co-assembled systems has been reported,^[37, 38] however, bioactive co-assembly has only previously been investigated in PA systems, with limited success,^[36] and in an Fmoc-SAP system where a non-gelating Fmoc-RGD molecule was used to decorate fibrils of Fmoc-FF.^[39] In each case, the mechanism by which the SAPs molecularly pack into the fibres is not well understood.

Here, we present a first step towards the development of truly co-assembled minimalist bioactive Fmoc-SAP scaffolds for the high-density presentation of multiple biochemical cues. In order to confirm the successful formation of co-assembled structures, we characterised the assembly process across all length scales; the molecular packing using Fourier transform infrared spectroscopy (FTIR), circular dichroism (CD) spectroscopy, and SAXS; the structures at longer length scales by transmission electron microscopy (TEM); and the bulk mechanical properties of the hydrogels by rheological measurements. We considered various co-assembled combinations of our two individual Fmoc-SAPs (Fmoc-FRGDF and Fmoc-DIKVAV) and distinguished the resulting co-assembled hydrogel structures (Figure 1c) from both single Fmoc-SAP and parallel mixed Fmoc-SAP systems (Figure 1d) to show that a unique homogenous nanofibrous hydrogel was obtained.

Results and Discussion

Self-Assembly of the Systems under Physiological Conditions

Titration of the single and co-assembled Fmoc-SAP systems (Figure 2a) was employed to determine the pK_a of the dual peptide system as a predictor of gelation behaviour. The pH switch-triggered self-assembly of these Fmoc-SAPs is correlated to the pK_a of the peptide,

which is dependent on the composition of the peptide chain.^[19] The titration curves for the single and co-assembled Fmoc-SAP systems displayed multiple pK_a shifts (inflection points on the curves in as seen in Figure 2a), corresponding to the formation of intermediary structures, with solution-gelation transition occurring at a pH in this region, when nanofibres predominate the system. In each case, significant pK_a shifts were observed between pH 7 and pH 9, indicating the ability of each system to form a stable hydrogel at physiological pH (~7.4), as was experimentally confirmed.^[19] These results indicate the flexibility of the gelation pH when tailoring a given system. Interestingly, the titration curve for the co-assembled FRGDF-DIKVAV appears as an average of the titration curves of the constituent single SAPs, Fmoc-FRGDF and Fmoc-DIKVAV, indicating a potential mechanism through which the gelation behaviour of co-assembled Fmoc-SAP systems can be tailored (Figure 2a). Overall, these titration curves demonstrate the pH neutrality of each Fmoc-SAP hydrogel, indicating the potential for these materials to be used for *in vitro* and *in vivo* applications.

Figure 2. (a) Titration curves showing shifts in pK_a for the individual peptides and FRGDF-DIKVAV combination. TEM images of (b) mixed and (c) co-assembled SAP hydrogels confirming nanofibre formation (scale = 200 nm).

Stable Nanofibrous Networks Retained Across Co-Assembled SAP Systems

Confident that self-assembly was occurring under physiological pH, the formation of the nanofibrous structures and the bundling of the networks for the mixed and co-assembled FRGDF-DIKVAV system was confirmed with negatively stained TEM (Figure 2b & c). As expected, the Fmoc-SAPs yielded nanofibres with consistent morphology and diameters, suggesting that the overall mechanism of nanofibre assembly was broadly maintained. The characteristic nanoscale assemblies observed for each of the single Fmoc-SAP systems were also prominent in the TEM micrographs of the mixed and co-assembled systems. Across both combined Fmoc-SAP systems, a stable nanofibrous network was maintained, with similar nanofibre dimensions observed in each case (Figure 2b & c).

The existence of comparable stable networks demonstrates the non-destructive nature of the hydrogel-mixing (Figure 2b) and that co-assembly resulted in nanoscale morphologies similar to the individual assemblies (Figure 2c), rather than allowing a secondary type of structure to form.^[40] However, while the nanoscale assemblies in each system resulted in the formation of nanofibres, morphological differences were observed between the hydrogel-mixed and co-assembled FRGDF-DIKVAV systems, suggesting that the combination method also plays a role in determining the overall network structure. Although drying effects must be considered, co-assembled FRGDF-DIKVAV displayed a lesser degree of nanofibre bundling than its mixed counterpart, with larger nanofibres distributed throughout the system. The co-assembled system contains nanofibres with width dimensions of $14.6 \text{ nm} \pm 1.9 \text{ nm}$ (mean $\pm$ standard deviation)(Figure 2b), which is statistically significantly different ($p = 0.0034$) from the mixed system nanofibres of $10.9 \text{ nm} \pm 1.9 \text{ nm}$ (Figure 2c). Furthermore, the greater standard deviation of the nanofibre widths in the mixed system (relative to the mean width) suggests inhomogeneity in the system.

Mechanical Properties of Hydrogels

For successful *in vivo* applications as adjuvant cell scaffolds, it is essential that the hydrogel scaffold closely mimics the native ECM both in terms of the chemical signal presented and the physical support provided.^[31] The elastic moduli of neural tissue ranges from 10^2 - 10^3 Pa while the moduli for connective and muscle tissues are $\sim 10^4$ Pa.^[41] As such, Fmoc-SAP hydrogels with elastic moduli in these ranges have the potential to act as adjuvant ECM-mimetic cell scaffolds with appropriate properties for tissue engineering applications.^[42] Therefore, it is important to determine the rheological properties of co-assembled Fmoc-SAP hydrogels. The rheological properties of hydrogels are controlled by a number of parameters, including nanofibre stiffness, concentration, branching and number/type of entanglements.^[43] Figure 3 shows the storage moduli (G') and loss moduli (G'') of the single, mixed, and co-assembled Fmoc-SAP systems as a function of applied frequency (Hz). All hydrogels displayed viscoelastic gel behaviour; with the storage moduli values higher than the loss moduli values with the range of frequencies measured.

Figure 3. Rheological data for single Fmoc-SAP sequences (a) FRGDF, (b) DIKVAV with comparison to (c) mixed and (d) co-assembled systems (G' = closed circles; G'' = open circles).

For the single Fmoc-SAP systems, the G' value of Fmoc-DIKVAV was at $\sim 10^4$ Pa (Figure 3b), in the appropriate range for application to tissue engineering, while the moduli for Fmoc-FRGDF are much lower (Figure 3a), as previously reported.^[19, 42] The G' value of the mixed FRGDF-DIKVAV system (Figure 3c) was similar to that of single Fmoc-DIKVAV, whereas the ratio of G''/G' (the $\tan \delta$ value) was similar to that of the single Fmoc-FRGDF system at 0.1-0.13 between 0.1-1 Hz. This suggests that the two Fmoc-SAPs co-existed as two separate non-interactive networks and both contributed to the overall rheological properties of the mixed system. In contrast, the moduli for co-assembled FRGDF-DIKVAV showed a $\sim 20\%$ reduction relative to Fmoc-DIKVAV, but the $\tan \delta$ was maintained (Figure 3d). This suggests

that co-assembled FRGDF-DIKVAV is a cooperative system with modification to the fibril interaction, which influenced the type/number of entanglements formed. Importantly, the rheological properties for co-assembled FRGDF-DIKVAV represent an intermediate between the weakest gel, Fmoc-FRGDF on one end and the strongest Fmoc-DIKVAV hydrogel on the other. This range of material properties suggests an as yet unidentified opportunity for controlling the stiffness of Fmoc-SAP hydrogels, where the combination of Fmoc-SAPs in particular ratios could achieve the tuning required for select tissue engineering applications.

Spectroscopic Analysis of Molecular Packing

In order to determine whether π - β assembly was the dominant structural mechanism driving the co-assembly of the Fmoc-SAP hydrogel systems spectroscopic analysis of bulk molecular interactions with both FTIR and CD were used. SAXS was then used to determine any differences between the structures of mixed and co-assembled systems on a fibrillar level.

FTIR spectra of each of the single, mixed, and co-assembled SAP systems are shown in Figure 4a. In each case, a major peak at approximately 1630 cm^{-1} and a minor peak at approximately 1690 cm^{-1} , indicating the presence of anti-parallel β -sheets, typical of this class of assembly.^[22] CD spectra for the single and mixed SAP systems similarly indicate the presence of β -sheets, with local maximum bands at approximately 190 nm and/or local minimum bands at approximately 218 nm (Figure 4b).^[44] However, the CD spectrum for the co-assembled system is inverted, a result of the π - π^* Cotton effect from the Fmoc moiety, implying an opposite chirality of the co-assembled system.^[45, 46] In addition, broad transitions in the 230 – 270 nm region for each of the systems (arising from numerous π - π^* transitions) indicate the supramolecular alignment of the peptide nanofibres into a hydrogel scaffold.^[19] The large transitions for the single Fmoc-FRGDF and Fmoc-DIKVAV hydrogels in this

region indicate a high degree of fibre bundling.^[33] The same effect is observed for the mixed and co-assembled SAP systems, though co-assembled FRGDF-DIKVAV displays smaller transitions relative to both the single and mixed SAP systems. This could be due to a reduced number of fibrils present due to a single population of heterogeneous structures.

Figure 4. (a) FTIR and (b) CD spectra for single, mixed, and co-assembled SAP hydrogels.

The transitions present in the region 180-220nm of the CD spectra for the single and mixed systems indicate the presence of β -sheets. On the other hand, the co-assembled FRGDF-DIKVAV system displayed a unique CD spectrum in this region, demonstrating an absorption shift of the minima from ~218 to 205nm, and a greatly reduced maxima ~190nm, an effect possibly arising from the novel interaction of aromatic and the carboxyl groups induced during the heterogeneous molecular packing of the penta- and hexapeptides^[47].

Structural Determination of SAP Assemblies using SAXS

Previous SAXS investigation of β -peptide oligomers indicated a good fit to a form factor model of a hollow cylinder with polydisperse radius to solutions of the β -peptide oligomers, suggesting that simple cylindrical models can provide suitable fits for β -peptide solutions.^[48]

To determine the most suitable form factor model for our systems, different models were

fitted to the experimental SAXS data, using fitting algorithms included in the NIST IgorPro software (Figure 5).

Figure 5. (a) Scattering data obtained using SAXS for FRGDF (blue) showing various cylindrical models fitted to data including cylinder (green), cylinder with polydisperse radius (purple), hollow cylinder (black), flexible cylinder (orange) and hollow cylinder with polydisperse radius (red). The hollow cylinder with polydisperse radius is the best fit for our SAXS data as highlighted in (b) at higher Q values for FRGDF. Similar fits were obtained using this model for (c) DIKVAV, (d) mixed and (e) co-assembled hydrogel systems (blue = SAXS experimental data; red = hollow cylinder with polydisperse radius model). For all cylindrical model fits for DIKVAV, DIKVAV/FRGDF mixed and co-assembled systems see Supplementary Figure 2.

TEM images were used to guide initial parameter values for the cylinder diameter and length, while the scattering length densities were adjusted to account for the use of these models for SAXS data (as the models were developed for small angle neutron scattering (SANS))

experiments).^[49] In each case, the optimal fit was achieved using the hollow cylinder with polydisperse radius form factor model (Figure 5). This result aligns with the previously observed model fitting for β -peptide oligomer solutions.^[48, 50] However, in our case, high quality fits were obtained without the need for associated structure factors.

Table 1. Hollow cylinder with polydisperse radius form factor model parameters for different peptide systems

Parameter	FRGDF	DIKVAV	Mixed FRGDF-DIKVAV	Co-Assembled FRGDF-DIKVAV
Length (Å)	3015	2979	2900	3315
Mean Core Radius (Å)	24.25	20.65	23.40	29.71
Radial Shell Thickness (Å)	29.52	28.17	27.06	51.88
Radial Polydispersity	0.06	0.10	0.19	0.12
SLD Core (Å⁻²)	1.31×10^{-5}	1.33×10^{-5}	1.36×10^{-5}	1.28×10^{-5}
SLD Shell (Å⁻²)	9.94×10^{-6}	9.91×10^{-6}	9.94×10^{-6}	9.76×10^{-6}
SLD Solvent (Å⁻²)	9.52×10^{-6}	9.49×10^{-6}	9.54×10^{-6}	9.49×10^{-6}
Chi-Squared (χ^2)	*4.40	1.62	1.88	1.02

*Though the χ^2 value here is high, this fit showed the best χ^2 value for FRGDF (see Supplementary Table 1)

The theoretical form factor model fit to the experimental data for each of the peptide systems enables physical properties of the peptide nanofibres to be determined (Table 1). In the single and mixed systems, the peptide nanofibres exhibit similar core and shell thicknesses of around 20-25 Å, and similar lengths, on the order of 3000 Å. However, for the co-assembled system, the mean core radius was increased to 29.7 Å and the radial shell thickness to 51.9 Å, closely agreeing with the nanofibre diameters observed in the TEM images (Figures 2b and 2c). These radial parameters (an increase in radial shell thickness of greater than 75% relative to the other peptide systems) are likely a result of the interaction of the two differently shaped and charged peptide sequences, requiring an increase in the radial shell thickness in order to achieve packing stability. Importantly, this suggests that the two SAPs are incorporated into a single structure, rather than forming a self-sorted heterogeneous system that would demonstrate properties closer to those of the mixed hydrogel. This result supports the

rheological measurements, where the co-assembled system was much weaker than the mixed or the Fmoc-DIKVAV systems, but stronger than the Fmoc-FRGDF, suggesting a distinct structure composed of the co-operative individual Fmoc-SAPs (Figure 3). The increase in nanofibre width highlights the potential for co-assembly to permit greater control over fibril morphology, which has not been previously attained in other SAP systems. Through altering the relative ratios of the different SAPs present in the co-assembled system, we can potentially alter the nanofibre width, allowing us to selectively tune fibril morphology for tissue-specific ECMs. The polydispersity in the cylinder (nanofibre) radius provides further confirmation of the formation of homogenous structures, with polydispersity values ranging between 0.06 and 0.12 for the single and co-assembled systems, indicating minimal variation in the cylinder radius along its length. However, as expected for the mixed system, the polydispersity value increases to 0.19, suggesting a greater degree of inhomogeneity arising from the presence of two separate populations of fibrils. It is also interesting to note that each of the peptide systems also displays higher scattering length density (SLD) values for the cylinder core than for either the cylinder itself, or the solvent, an effect that could be due to the absorption of salt from the PBS solution into the centre of the nanofibre cylinder.

This data underlies the importance of deeper analysis of two-component systems. In terms of the bulk properties, both the mixed and co-assembled systems appear quite similar to the individual FRGDF and DIKVAV systems, as is expected. Despite this, the SAXS results demonstrate clear differences in the structure of the co-assembled system highlighting its usefulness as a technique for analysing multicomponent systems.

Conclusions

In summary, novel co-assembled Fmoc-SAP systems were developed with the potential to present multiple bioactive epitopes and synergistically provide appropriate physical and chemical cues for cellular regeneration and repair. We demonstrate that multiple signals can be included into a nanofibrillar SAP hydrogel by mixing two preformed gels, or by co-assembling two distinct SAPs. In particular, we show for the first time the successful co-assembly of multiple bioactive SAP sequences into a single homogeneous nanofiber scaffold. This process enables us to produce a tuneable biomaterial that can include multiple bioactive sequences in precise ratios. Importantly, the observed increase in the width of the co-assembled nanofibres demonstrates a potential avenue for the tuning of fibril morphology through the co-assembly of multiple SAP systems, indicating the capacity for co-assembly to enable tuning of biomimetic SAP scaffolds to the ECM of particular tissues. This study describes the potential for a single nanofibrous system to concomitantly present these signals to individual cells, providing a suitable ECM-mimetic material for the complex coordination of cell fate. This co-assembled system therefore provides a platform for an enhanced biomimetic material for future *in vitro* and *in vivo* tissue engineering applications.

Methods

Solid Phase Peptide Synthesis (SPPS): Fmoc-SAPs were made using solid phase peptide synthesis (SPPS)^[51] in a custom rotating glass reactor vessel at a 0.4 mmol scale. Fmoc protected amino acids, Hydroxybenzotriazole (HOBt), O-Benzotriazole-N,N,N',N'-tetramethyl-uronium-hexafluoro-phosphate (HBTU) and Wang based resins were purchased from GL Biochem (China). All other chemicals were purchased from Sigma-Aldrich.

Dimethylformamide (DMF) was dried for a minimum of 2 hours prior to use with 4 Å molecular sieves.

Peptide synthesis was achieved by the stepwise deprotection of the N-terminal Fmoc group of the resin anchored amino acid using a solution of 20% piperidine in DMF for 20 minutes, followed by a coupling step using a solution of the next Fmoc-amino acid in 2.0 mmol HoBT, 1.9 mmol HBTU and 4.8 mmol N,N-diisopropylethylamine (DIPEA) in DMF for 60 minutes. This process of deprotection and coupling was repeated until the desired peptide was synthesised. The final Fmoc group was not removed. Deprotection and coupling of amino acids was verified using a Kaiser test for the detection of free amines.^[51] Once SPPS of the desired sequence was complete, the Fmoc protected peptide and resin was washed with ethanol and left to dry under constant vacuum for 2 days. Once complete, a cleavage solution of trifluoroacetic acid (TFA), 2.5% deionised water (DI H₂O) and 2.5% triethylsilane (TES) was prepared. The resin was left to stand in the cleavage solution for 2 hours. Following cleavage, the peptide solution was filtered through glass wool. Excess TFA was then evaporated using nitrogen until approximately 5 mL of peptide solution was left. The peptide solution was then precipitated, followed by 5 washes in cold ether before being collected and allowed to dry under constant vacuum for 2 days. The peptide was then ground into a fine powder before being placed under constant vacuum for a further 7 days.

Single Fmoc-SAP Hydrogel Preparation: Peptide hydrogels were prepared at concentrations of 10 and 20 mg/mL. Prior to peptide preparation, 10 mL of phosphate buffered saline (PBS) solution was prepared and the pH noted. For peptide preparation, 5 mg of peptide was weighed and placed in an eppendorf vial. 50 µL of deionised water (DI H₂O) was added dropwise to the vial while the solution was vortexed. Next, 25 µL of 0.5 M sodium hydroxide (NaOH) was similarly added dropwise, and if necessary sonication of the solution was performed using an Eumax UD80SH-2L ultrasonic cleaner at room temperature for periods

of between 2 and 3 minutes until full dissolution of the peptide. Gelation was achieved via the dropwise addition of 0.1 M hydrochloric acid (HCl) over vortex. As this process continued, the hydrogel pH was measured using an Oaktron pH 700 micro pH electrode (Thermo Scientific) until a stable hydrogel at pH slightly above the PBS pH was reached. Finally, PBS was added dropwise to yield a total solution of 500 μ L for a concentration of 10 mg/mL or until a total solution of 250 μ L for a concentration of 20 mg/mL. Mass spectrometry was performed to confirm the desired peptide sequence was synthesised (Supplementary Figure 1).

Mixed Fmoc-SAP Hydrogel Preparation: The mixed Fmoc-SAP hydrogel consisted of two different single Fmoc-SAP hydrogels, Fmoc-FRGDF and Fmoc-DIKVAV. In each case, the single Fmoc-SAP hydrogels were prepared as detailed above. The mixed Fmoc-SAP hydrogel was then prepared through the combination of the single Fmoc-SAP hydrogels over vortex. Single Fmoc-SAP hydrogels were combined in volumetrically equal ratios, with vortex mixing used to combine the single Fmoc-SAP hydrogels.

Co-Assembled Fmoc- SAP Hydrogel Preparation: The co-assembled Fmoc-SAP hydrogel was prepared in a similar manner to the single Fmoc-SAP hydrogels, however the single peptide used in hydrogel preparation was replaced with two different peptides, Fmoc-FRGDF and Fmoc-DIKVAV. These peptides were combined in equal mass ratios to a total mass of 5 mg. Hydrogel formation was then achieved as detailed in section above.

Transmission Electron Microscopy: Negative stain TEM was performed using a 125 kV Hitachi H7100FA electron microscope with a LaB₆ cathode. Formvar coated copper grids were prepared and electron glow-discharged for 30 seconds at 15 mA. A piece of parafilm was prepared with a 20 μ L drop of hydrogel sample (prepared as above), two 20 μ L drops of DI H₂O and two drops of uranyl formate (UF) solution (0.75%). The Formvar-coated side of

the grid was applied to the hydrogel sample for 30 seconds before excess was blotted off using filter paper. The same technique was used for each drop of water (3 seconds each) and each drop of UF (3 seconds followed by 20 seconds)^[52]. Any excess stain solution was blotted off and the grid was allowed to dry overnight before imaging in the TEM. Nanofibre dimensions for the mixed and co-assembled systems were calculated based on the TEM images using ImageJ. A selection of 10 width measurements across different nanofibres were taken and a two-tailed T-test used to compare the mean width values between co-assembled and mixed systems.

Titration: 50 μL of 1 M NaOH was added dropwise to a vial containing 10 mg of peptide while mixed over vortex. Next, 200 μL of DI H_2O was similarly added dropwise over vortex. The pH of the solution was measured using an Oaktron pH 700 micro pH electrode (Thermo Scientific). 10 μL of 0.2 M HCl was added dropwise over vortex until the hydrogel solution reached pH 3. pH was measured after addition of each 10 μL of HCl.

Rheology: Rheological analysis was performed using a Kinexus Pro+ Rheometer (Malvern). Approximately 0.5 mL of hydrogel sample was placed on a flat plate geometry with solvent trap (Upper Geometry: PU20 SR1351 SS, Lower Geometry: PLS55 C0177 SS). A gap distance of 0.2 mm was used and multiple frequency sweeps were performed for frequencies ranging from 0.1–100 Hz with a 0.1% strain at a constant temperature of 25°C.

Fourier Transform Infrared Spectroscopy: FTIR was completed using an Alpha Platinum ATR FTIR (Bruker Optics). Approximately 100 μL of peptide hydrogel was placed on the single reflection diamond and the pressure applicator used to adequately spread hydrogel over crystal surface. Absorbance scans of the amide I region (1550 to 1750 cm^{-1}) were obtained for each peptide, and a background buffer scan subtracted.

Circular Dichroism: CD was completed using a Chirascan CD Spectrometer (Applied Photophysics Limited). 1000 μL of peptide hydrogel solutions were prepared in 1:200 ratios with DI H_2O . Using a 400 μL cuvette with a 10 mm pathlength, a baseline scan of DI H_2O was completed and subtracted from the following peptide CD scans. The cuvette was filled with 400 μL of peptide solution and a CD scan from 180 nm to 320 nm with a step size of 0.5 and 1 nm bandwidth was obtained. The resulting data was averaged and smoothed post-acquisition using software provided by Chirascan prior to analysis.

Small Angle X-Ray Scattering: SAXS was performed using the SAXS/WAXS beamline at the Australian Synchrotron. Measurements were taken using two different camera lengths: 900 mm and 7000 mm with an X-ray wavelength of 1.0332 Å. These camera lengths enabled the scattering vector (q) to be measured across the range 0.008 to 0.7 Å⁻¹. The diffraction pattern was recorded on a Pilatus 1M detector (170 mm x 170 mm, effective pixel size of 172 μm x 172 μm), and processed using the Australian Synchrotron ScatterBrain software.

Hydrogel samples were prepared, as detailed above, two days prior to testing and stored in eppendorf vials. Prior to sample exposure, PBS backgrounds were loaded into 1.5 mm glass capillaries and sealed to prevent evaporation. Capillaries were then loaded into a custom mount designed to enable movement of the capillaries in two dimensions. Two 5-second exposures were taken for each sample background using the two different camera lengths. PBS backgrounds were then removed from the capillaries and replaced with hydrogel samples. Capillaries were then resealed and remounted for measurement. Multiple 5-second exposures were taken for each sample on the two different camera lengths at different positions along the capillary length.

Repeated measurements for each sample on each camera length were averaged and the PBS background subtracted using ScatterBrain. q calibration was performed using air shot

scattering curves, though the use of capillaries prevented standard intensity normalisation. To circumvent this issue, intensity normalisation was performed relative to a known scattering curve for de-fatted human serum albumin (DFHSA) at a protein concentration of 1 mg/mL. Due to weak scattering at higher q values, the background was scaled by 0.9 prior to subtraction from the sample scattering data. Data for each sample on the two different camera lengths were then combined using IgorPro, though data at the lower q range (obtained using the 7000 mm camera length) showed a linear scattering curve for all samples (likely due to aggregation) and so was discarded. Analysis of the scattering curves for each sample was performed across the q range 0.01 to 0.3 Å⁻¹. The intensity of the x-rays scattered from each sample, $I(q)$, was then compared to a number of theoretical form factors, $F(q)$, which result from the particle geometry. $I(q)$ can be described as the product of the number density of scattered objects (n_p), the form factor ($F(q)$), and the structure factor ($S(q) = 1$, for our dilute system), which describes interparticle interactions (Eq. 1).

$$I(q) = n_p F(q) S(q) \quad (1)$$

$I(q)$ for each of our samples was compared to form factor models provided by the National Institutes of Standards and Technology (NIST) for use in conjunction with IgorPro, with the quality of the fit estimated using a chi-square test.^[49] These models included calculations of n_p , though structure factors had to be calculated separately. While structure factors are widely used to generate higher quality models of x-ray scattering intensity, IgorPro applies an average structure factor approximation, which rests on the assumption of homogeneous, monodisperse, spherical particles. As our samples consist of inhomogeneous, polydisperse, non-spherical particles, the use of an average structure approximation is inappropriate. Though structure factors for our samples could be calculated using polymer reference interaction site model (PRISM) theory, high-quality fits were obtained without the reliance on structure factors and they have hence been excluded from our calculations of $I(q)$.^[53, 54]

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References

1. Holmes, T. C.; de Lacalle, S.; Su, X.; Liu, G.; Rich, A.; Zhang, S., *Proceedings of the National Academy of Sciences* **2000**, 97 (12), 6728-6733.
2. Nisbet, D. R.; Williams, R. J., *Biointerphases* **2012**, 7 (1-4), 2. DOI 10.1007/s13758-011-0002-x.
3. Pettikiriarachchi, J. T. S.; Parish, C. L.; Schoichet, M. S.; Forsythe, J. S.; Nisbet, D. R., *Australian Journal of Chemistry* **2010**, 63, 1143-1154.
4. Drury, J. L.; Mooney, D. J., *Biomaterials* **2003**, 24 (24), 4337-4351. DOI 10.1016/s0142-9612(03)00340-5.
5. Hynd, M. R.; Turner, J. N.; Shain, W., *Journal of biomaterials science. Polymer edition* **2007**, 18 (10), 1223-44. DOI 10.1163/156856207782177909.
6. Atala, A.; Mooney, D. J., *Synthetic biodegradable polymer scaffolds*. Springer: **1997**.
7. Ghasemi-Mobarakeh, L.; Prabhakaran, M. P.; Morshed, M.; Nasr-Esfahani, M. H.; Ramakrishna, S., *Biomaterials* **2008**, 29 (34), 4532-9. DOI 10.1016/j.biomaterials.2008.08.007.
8. Nisbet, D. R.; Rodda, A. E.; Horne, M. K.; Forsythe, J. S.; Finkelstein, D. I., *Tissue engineering. Part A* **2010**, 16 (9), 2833-42. DOI 10.1089/ten.TEA.2009.0677.
9. Monteiro, N.; Riberio, D.; Martins, A.; Faria, S.; Fonesca, N. A.; Moreira, J. N.; Reis, R. L.; Neves, N. M., *ACS Nano* **2014**, 8 (8), 8082-8094.
10. Peppas, N. A.; Keys, K. B.; Torres-Lugo, M.; Lowman, A. M., *Journal of Controlled Release* **1999**, 62, 81-87.
11. Tsai, E. C.; Dalton, P. D.; Schoichet, M. S.; Tator, C. H., *Journal of Neurotrauma* **2004**, 21 (6), 789-804.

12. Suri, S.; Schmidt, C. E., *Tissue Engineering: Part A* **2010**, *16* (5), 1703-1716.
13. Carone, T. W.; Hasenwinkel, J. M., *Journal of biomedical materials research. Part B, Applied biomaterials* **2006**, *78* (2), 274-82. DOI 10.1002/jbm.b.30483.
14. Li, X.; Xiao, Z.; Han, J.; Chen, L.; Xiao, H.; Ma, F.; Hou, X.; Li, X.; Sun, J.; Ding, W.; Zhao, Y.; Chen, B.; Dai, J., *Biomaterials* **2013**, *34* (21), 5107-16. DOI 10.1016/j.biomaterials.2013.03.062.
15. Liang, Y.; Walczak, P.; Bulte, J. W., *Biomaterials* **2013**, *34* (22), 5521-9. DOI 10.1016/j.biomaterials.2013.03.095.
16. Ma, P. X., *Materials Today* **2004**, *7* (5), 30-40. DOI 10.1016/s1369-7021(04)00233-0.
17. Wu, E. C.; Zhang, S.; Hauser, C. A. E., *Advanced Functional Materials* **2012**, *22* (3), 456-468. DOI 10.1002/adfm.201101905.
18. Zhang, S., *Nature biotechnology* **2003**, *21* (10), 1171-8. DOI 10.1038/nbt874.
19. Rodriguez, A. L.; Parish, C. L.; Nisbet, D. R.; Williams, R. J., *Soft Matter* **2013**, *9* (15), 3915. DOI 10.1039/c3sm27758e.
20. Gelain, F.; Horii, A.; Zhang, S., *Macromolecular bioscience* **2007**, *7* (5), 544-51. DOI 10.1002/mabi.200700033.
21. Lakshmanan, A.; Zhang, S.; Hauser, C. A., *Trends in biotechnology* **2012**, *30* (3), 155-65. DOI 10.1016/j.tibtech.2011.11.001.
22. Smith, A. M.; Williams, R. J.; Tang, C.; Coppo, P.; Collins, R. F.; Turner, M. L.; Saiani, A.; Ulijn, R. V., *Advanced Materials* **2008**, *20* (1), 37-41. DOI 10.1002/adma.200701221.
23. Jayawarna, V.; Ali, M.; Jowitt, T. A.; Miller, A. F.; Saiani, A.; Gough, J. E.; Ulijn, R. V., *Advanced Materials* **2006**, *18* (5), 611-614. DOI 10.1002/adma.200501522.
24. Kim, S.; Kim, J. H.; Lee, J. S.; Park, C. B., *Small* **2015**. DOI 10.1002/smll.201500169.
25. Burch, R. M.; Weitzberg, M.; Blok, N.; Muhlhauser, R.; Martin, D.; Farmer, S. G.; Bator, J. M.; Connor, J. R.; Ko, C.; Kuhn, W.; McMillan, B. A.; Raynor, M.; Shearer, B. G.; Tiffany, C.; Wilkins, D. E., *Proceedings of the National Academy of Sciences* **1991**, *88*, 355-359.
26. Silva, G. A.; Czeisler, C.; Niece, K. L.; Beniash, E.; Harrington, D. A.; Kessler, J. A.; Stupp, S. I., *Science* **2004**, *303* (5662), 1352-5. DOI 10.1126/science.1093783.
27. Anderson, J. M.; Andukuri, A.; Lim, D. J.; Jun, H.-W., *ACS Nano* **2009**, *3* (11), 3447-3454.
28. Cormier, A. R.; Pang, X.; Zimmerman, M. I.; Zhou, H.-X.; Paravastu, A. K., *ACS Nano* **2013**, *7* (9), 7562-7572.
29. Berns, E. J.; Sur, S.; Pan, L.; Goldberger, J. E.; Suresh, S.; Zhang, S.; Kessler, J. A.; Stupp, S. I., *Biomaterials* **2014**, *35* (1), 185-95. DOI 10.1016/j.biomaterials.2013.09.077.
30. Lau, C. L.; Kovacevic, M.; Tingleff, T. S.; Forsythe, J. S.; Cate, H. S.; Merlo, D.; Cederfur, C.; Maclean, F. L.; Parish, C. L.; Horne, M. K.; Nisbet, D. R.; Beart, P. M., *Journal of neurochemistry* **2014**, *130* (2), 215-26. DOI 10.1111/jnc.12702.
31. Matson, J. B.; Stupp, S. I., *Chemical communications* **2012**, *48* (1), 26-33. DOI 10.1039/c1cc15551b.

32. Tovar, J. D.; Rabatic, B. M.; Stupp, S. I., *Small* **2007**, 3 (12), 2024-8. DOI 10.1002/smll.200600645.
33. Modepalli, V. N.; Rodriguez, A. L.; Li, R.; Pavuluri, S.; Nicholas, K. R.; Barrow, C. J.; Nisbet, D. R.; Williams, R. J., *Biopolymers* **2014**, 102 (2), 197-205. DOI 10.1002/bip.22469.
34. Rodriguez, A. L.; Wang, T. Y.; Bruggeman, K. F.; Horgan, C. C.; Li, R.; Williams, R. J.; Parish, C. L.; Nisbet, D. R., *Journal of Materials Chemistry B* **2014**, 2, 7771-7778.
35. Gelain, F.; Silva, D.; Caprini, A.; Taraballi, F.; Natalello, A.; Villa, O.; Nam, K. T.; Zuckermann, R. N.; Doglia, S. M.; Vescovi, A., *ACS Nano* **2011**, 5 (3), 1845-1859.
36. Niece, K. L.; Hartgerink, J. D.; Donners, J. J. J. M.; Stupp, S. I., *Journal of the American Chemical Society* **2003**, 125, 7146-7147.
37. Buerkle, L. E.; Rowan, S. J., *Chemical Society reviews* **2012**, 41 (18), 6089-102. DOI 10.1039/c2cs35106d.
38. Xavier, J. R.; Thakur, T.; Desai, P.; Jaiswal, M. K.; Sears, N.; Cosgriff-Hernandez, E.; Kaunas, R.; Gaharwar, A. K., *ACS Nano* **2015**, 9 (3), 3109-3118.
39. Zhou, M.; Smith, A. M.; Das, A. K.; Hodson, N. W.; Collins, R. F.; Ulijn, R. V.; Gough, J. E., *Biomaterials* **2009**, 30 (13), 2523-30. DOI 10.1016/j.biomaterials.2009.01.010.
40. Foster, J. A.; Edkins, R. M.; Cameron, G. J.; Colgin, N.; Fucke, K.; Ridgeway, S.; Crawford, A. G.; Marder, T. B.; Beeby, A.; Cobb, S. L.; Steed, J. W., *Chemistry* **2014**, 20 (1), 279-91. DOI 10.1002/chem.201303153.
41. Saha, K.; Keung, A. J.; Irwin, E. F.; Li, Y.; Little, L.; Schaffer, D. V.; Healy, K. E., *Biophysical journal* **2008**, 95 (9), 4426-38. DOI 10.1529/biophysj.108.132217.
42. Li, R.; Horgan, C. C.; Long, B.; Rodriguez, A. L.; Mather, L.; Barrow, C. J.; Nisbet, D. R.; Williams, R. J., *RSC Adv.* **2015**, 5 (1), 301-307. DOI 10.1039/c4ra13266a.
43. Raeburn, J.; Zamith Cardoso, A.; Adams, D. J., *Chemical Society reviews* **2013**, 42 (12), 5143-56. DOI 10.1039/c3cs60030k.
44. Greenfield, N. J., *Nature protocols* **2006**, 1 (6), 2876-90. DOI 10.1038/nprot.2006.202.
45. Charney, E.; Ziffer, H.; Weiss, U., *Tetrahedron* **1965**, 21, 3121-3126.
46. Gaffield, W., *Tetrahedron* **1970**, 26, 4093-4108.
47. Velluz, L.; Legrand, M.; Grosjean, M., *Optical circular dichroism*. Academic Press: **1965**.
48. Pizzey, C. L.; Pomerantz, W. C.; Sung, B. J.; Yuwono, V. M.; Gellman, S. H.; Hartgerink, J. D.; Yethiraj, A.; Abbott, N. L., *The Journal of chemical physics* **2008**, 129 (9), 095103. DOI 10.1063/1.2955745.
49. Kline, S. R., *Journal of Applied Crystallography* **2006**, 39 (6), 895-900. DOI 10.1107/s0021889806035059.
50. Abul-Haija, Y. M.; Roy, S.; Frederix, P. W. J. M.; Javid, N.; Jayawarna, V.; Ulijn, R. V., *Small* **2014**, 5, 973-979. DOI 10.1002/smll201301668.
51. Amblard, M.; Fehrentz, J.-A.; Martinez, J.; Subra, G., *Molecular Biotechnology* **2006**, 33, 239-254.

52. Booth, D. S.; Avila-Sakar, A.; Cheng, Y., *Journal of visualized experiments : JoVE* **2011**, (58). DOI 10.3791/3227.
53. Curro, J. G.; Schweizer, K. S., *Macromolecules* **1987**, 20, 1928-1934.
54. Schweizer, K. S.; Yethiraj, A., *The Journal of chemical physics* **1993**, 98 (11), 9053. DOI 10.1063/1.464465.

Supplementary information

Supplementary Figure 1. Mass spectra for (a) Fmoc-DIKVAV and (b) Fmoc-FRGDF.

Supplementary Figure 2. Scattering data (blue) obtained using SAXS for (a) DIKVAV, (b) FRGDF-DIKVAV mixed and (c) co-assembled systems. Various cylindrical models fitted to data are shown including cylinder (green), cylinder with polydisperse radius (purple), hollow cylinder (black), flexible cylinder (orange) and hollow cylinder with polydisperse radius (red). The hollow cylinder with polydisperse radius is the best fit for our SAXS data as highlighted at higher Q values for (d) DIKVAV, (e) FRGDF-DIKVAV mixed and (f) co-assembled systems.

Supplementary Table 1. Chi-squared (χ^2) values for the various form factor model fits to the experimental data for each SAP system. The optimal fit (bold) for each system is found with the hollow cylinder with polydisperse radius model.

Form Factor Model	FRGDF	DIKVAV	Mixed FRGDF-DIKVAV	Co-Assembled FRGDF-DIKVAV
Cylinder	26.23	7.55	11.19	28.89
Cylinder (Polydisperse Radius)	12.38	1.72	4.63	8.64
Hollow Cylinder	21.83	1.71	2.88	3.52
Flexible Cylinder	12.11	3.28	4.99	17.40
Hollow Cylinder (Polydisperse Radius)	4.40	1.62	1.88	1.02