Impact of Gelation Method on Thixotropic Properties of Phenylalanine-Derived Supramolecular Hydrogels

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Abstract

Supramolecular hydrogels formed by noncovalent self-assembly of low molecular weight (LMW) agents are promising next-generation biomaterials. Thixotropic shear response and mechanical stability are two emergent properties of hydrogels that are critical for biomedical applications including drug delivery and tissue engineering in which injection of the hydrogel will be necessary. Herein, we demonstrate that the emergent thixotropic properties of supramolecular phenylalanine-derived hydrogels are dependent on the conditions in which they are formulated. Specifically, hydrogels formed from fluorenylmethoxycarbonyl (Fmoc) modified phenylalanine derivatives, 3-fluorophenylalanine (Fmoc-3F-Phe) and pentafluorophenylalanine (Fmoc-F5-Phe), were characterized as a function of gelation conditions to examine how shear response and mechanical stability properties correlate to mode of gelation. Two distinct methods of gelation were compared. First, spontaneous self-assembly and gelation was triggered by a solvent exchange method in which a concentrated solution of the gelator in dimethylsulfoxide was diluted into water. Second, gelation was promoted by dissolution of the gelator into water at basic pH followed by gradual pH adjustment from basic to mildly acidic by the hydrolysis of glucono-delta-lactone. Hydrogels formed under solvent exchange conditions were mechanically unstable and poorly shear-responsive whereas hydrogels formed by gradual acidification were temporally stable and had highly shear-responsive viscoelastic character. These studies confirm that gelation environment and mechanism have a significant influence on the emergent properties of supramolecular hydrogels and offer insight into how gelation conditions can be used to tune hydrogel properties for specific applications.

Introduction

Supramolecular hydrogels are formulated through the noncovalent self-assembly of gelators into one-dimensional fibrils or worm-like micelles, which then entangle to establish a hydrogel network.[1-3] This self-assembly arises from noncovalent contacts including hydrophobic, Coulombic, hydrogen bonding, and aromatic π-π interactions.[4-7] Supramolecular hydrogels, which are often composed of bioderived molecules, have advantages as biomaterials compared to covalent polymer hydrogels due to enhanced biocompatibility with cells and tissues both *in vitro* and *in vivo.*[8-11] Many supramolecular hydrogels also exhibit superior viscoelastic and biochemical properties that position them to be excellent next-generation materials for biological applications. Thixotropic shear-responsive self-healing behavior is an important viscoelastic property of hydrogels for biological applications like drug delivery and tissue engineering since these applications ideally require the hydrogel and any encapsulated cargo to be delivered by injection.[12-14] Injectable hydrogels must thin upon application of shear forces, as would occur when forcing the gel through a needle, and quickly recover after the shear force is removed.[13,15-16]

Self-assembled peptides have been engineered to form hydrogel networks that have been broadly exploited for biological applications such as tissue engineering, drug delivery, hemostasis, and as antimicrobials.[3,10,17-22] Peptide-derived hydrogels have been reported that exhibit all the requisite emergent properties, including shear-responsive behavior, for these types of sophisticated biological applications. However, the high cost of producing synthetic peptides on a practically useful scale has impeded the widespread use of supramolecular peptide hydrogels.[23] Low molecular weight (LMW) supramolecular gelators, often defined as molecules with a molecular mass of less than 500 Da, have drawn intense interest as cost-efficient

alternatives to peptides as supramolecular hydrogel biomaterials.[24-25] Gels formed from the self-assembly of LMW gelators have now been developed which exhibit comparable properties to those formed from self-assembled peptides and covalently linked polymers.[26-29] The most promising of these LMW gelators include short peptides and modified amino acids.[30-39] Fluorenylmethoxycarbonyl (Fmoc) modified amino acids, particularly those with aromatic sidechains such as phenylalanine and tyrosine, are a promising class of LMW hydrogel.[7,40-50] However, tuning the emergent properties of these LMW has been challenging compared to longer self-assembling peptides due to the limited chemical space available for modification.[11,51-55] Thus, Fmoc-amino acids remain the subject of research efforts focused on understanding and optimizing their emergent viscoelastic and biochemical properties.

The thixotropic shear-responsive behavior demonstrated by Fmoc-amino acid supramolecular hydrogels has proven to be idiosyncratic and challenging to modify.[56] There is a need to more fully understand the fundamental physiochemical parameters that result in shear-responsive self-healing in order to exploit these materials for applications in tissue engineering and drug delivery. Adams and coworkers have shown that hydrogel formulation method can exert a profound impact on the emergent properties of hydrogels formed from LMW dipeptide gels.[57-59] Accordingly, we herein report a systematic study of the influence of gelation conditions on the physical properties of Fmoc-amino acid derivatives. Specifically, we have explored the emergent thixotropic properties of Fmoc-Phe derivatives, including Fmoc-3-fluorophenylalanine (Fmoc-3F-Phe) and Fmoc-pentafluorophenylalanine (Fmoc-F5-Phe) (**Figure 1**).[40,60] There are two prominent methods that have been used to initiate assembly and gelation of these LMW gelators: solvent exchange[24,40,58,61-63] and gradual pH adjustment.[64-66] The solvent exchange method exploits the differing solubility of gelators in the organic solvent; upon dilution into

water the gelator undergoes assembly and gelation. The pH adjustment method takes advantage of the acidic carboxylic acid proton to solubilize the gelator under basic conditions; gradual protonation upon acidification initiates assembly and gelation.[64-65] When comparing hydrogels assembled using the two distinct methods, the gels formed by solvent exchange exhibited poor shear-responsive properties and were mechanically unstable, whereas hydrogels assembled using the gradual pH adjustment showed increased stability and high shear-responsiveness. The studies herein confirm that the method and conditions of gelation significantly change the emergent properties of supramolecular hydrogels and can offer a further avenue to tune these properties for specific applications.

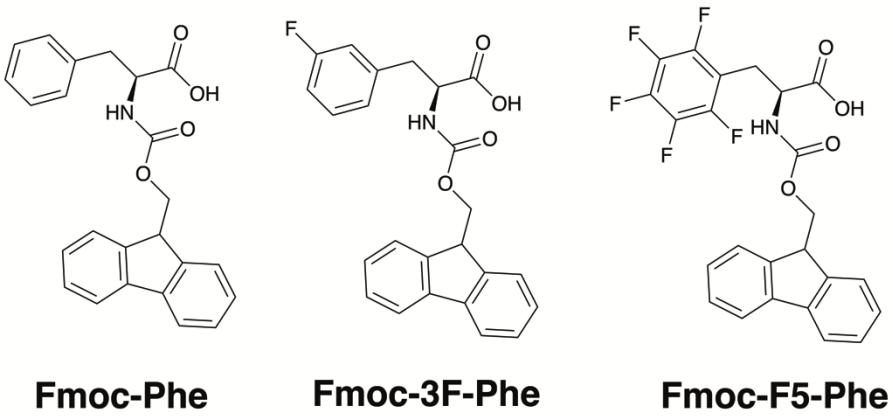


Figure 1. Chemical structures of Fmoc-phenylalanine derivatives.

Materials and Methods

Materials

Reagents and organic solvents were purchased commercially and used without further purification. Fmoc-phenylalanine derivatives were purchased at the highest available commercial purity and then used in gelation experiments without further purification. Water was purified by filtration prior to use (0.2 μm filter, 18 MΩ).

Hydrogelation by solvent exchange

For self-assembly and hydrogelation via DMSO solvent-exchange, Fmoc- amino acid gelators were dissolved in dimethyl sulfoxide (DMSO) at 247 mM. To form hydrogels, this DMSO stock solution was diluted into water to give a final concentration of 5 mM gelator (2% DMSO/H_2O *v/v*). The dilution was gently mixed via pipette, briefly agitated with a vortex mixer, and then left undisturbed to allow gelation. As described previously,[7,34,40,56,60,67-70] the solutions were opaque suspensions immediately after dilution and became transparent hydrogels after 1–5 minutes, depending on the gelator used. Hydrogels reached their most stable hydrogel form after roughly three hours. DMSO was chosen as the organic solvent for these studies based on previous studies that have demonstrated its convenient use to promote gelation by solvent exchange.[7,34,40,56,60,67-70]

Hydrogelation by pH adjustment

For hydrogelation by pH adjustment by glucono-δ-lactone (GdL) hydrolysis, Fmoc-amino acid gelators were dissolved in water at concentrations of 5 or 15 mM using 0.1 M NaOH at a 1:1 molar equivalent to aid in dissolution.[42,57,65,71-72] A fresh solution of GdL was prepared at a concentration of 100 mg mL^{-1} (561 mM) immediately prior to gelation. Hydrogels were prepared by adding 1 molar equivalent of the GdL solution into the amino acid solution (9 μL of GdL solution for 1 mL of a 5 mM hydrogel and 27 μL of GdL solution for 1 mL of a 15 mM hydrogel, respectively). The solution was mixed by brief agitation by vortex after additional of GdL and left undisturbed for approximately 12 h to allow gelation.

Transmission electron microscopy

Transmission electron microscopy images were acquired using a Hitachi 7650 transmission electron microscope with an accelerating voltage of 80 kV. Hydrogels (10 μL) were applied directly onto 100 mesh carbon-coated copper grids and allowed to stand for 1 minute before being removed by capillary action. The grids were then stained with 10 μL of uranyl acetate for 10 minutes before this too was removed by capillary action. The grids were allowed to dry prior to imaging.

Oscillatory Rheology

Oscillatory rheology was conducted using a TA instruments Discovery HR-2 rheometer. A 20 mm parallel plate geometry was used for the experiments. The gap was set individually for each experiment to accommodate inconsistencies in the size of the hydrogel sample on the plate. Strain sweep analyses were first conducted using a constant frequency of 1 Hz and varying percent strain from 0.1% to 100% in order to determine the linear viscoelastic region for each gel (see Supplementary Information, Figures S1–S6). Solvent exchange method hydrogels were formed in two ways. First, they were allowed to form by depositing the suspension formed immediately after dilution from DMSO into water on the rheometer stage and gelation occurred *in situ* on the stage under strain and was followed by a dynamic time sweep. Second, the hydrogels were formed in tubes and allowed to mature for 3 hours before transfer of the gels to the rheometer stage for analysis (see Supplementary Information, Figure S7). In order to ensure that the transferred gels provided reliably representative rheological data, it was necessary to transfer the hydrogels very cautiously and maintain very precise placement of the hydrogel sample on the rheometer stage as well as identical hydrogel volumes for the different samples. These different gelation methods produced differing viscoelastic properties in the resulting

hydrogels, as described in the Discussion section. For the former methods, a 1 mL suspension of the gelator diluted from DMSO into water was formed in an Eppendorf tube and briefly mixed before 500 μL were transferred to the rheometer stage after which the gap was set. With this information, we then conducted dynamic frequency sweep experiments to determine the storage (G′) and loss (G″) moduli for each hydrogel. Frequency sweep experiments were conducted from 0.1–100 rad s^{-1} at 1% strain at 25 ºC (see Supplementary Information, Figures S8–S11. These data are consistent with previously reported viscoelastic determinations for these hydrogels.[40,56,60,73-74]

The thixotropic viscoelastic properties of the hydrogels were determined by dynamic time sweep experiments that indicated shear recovery after applied strain. The dynamic time sweep for these hydrogels were conducted at a constant frequency of 1 Hz (6.28 rad s^{-1}). The strain was held at 0.2% for 85 minutes, followed by a period of 100% strain which lasted 2.5 minutes, then another period of 0.2% strain which lasted for 60 minutes, another period of 100% strain for 5 minutes, and finally a period at 0.2% strain for 60 minutes. Hydrogels made through pH adjustment were not formed on plate, as their formation occurred much more slowly. Hydrogels (1 mL) were formed in Eppendorf tubes and allowed to mature for 24 hours. The tubes were then carefully cut at the 0.5 mL mark and the top portion of the hydrogel was transferred to the rheometer plate. The dynamic time sweeps for hydrogels of this type were similar, but shorter as they recover much more rapidly. The frequency was held constant at 1 Hz. Strain began at 0.2% for five minutes, followed by a 2.5-minute period of 100% strain, then 0.2% strain for five minutes, then 100% strain for five minutes, and finally 0.2% strain for five minutes. All dynamic time sweep experiments were performed in triplicate on separate hydrogel samples and all plotted data pointes are presented as the average of the three experiments.

Results

Self-healing thixotropic properties of the hydrogels on the bulk scale

In this study, we interrogated the thixotropic properties of hydrogels of two Fmoc-Phe derivatives under two comparative gelation conditions. Fmoc-3F-Phe and Fmoc-F5-Phe (**Figure 1**) were chosen as representative Phe-derived LMW supramolecular hydrogel systems. We have previously shown that halogenation, and in particular, fluorination, has a dramatic impact in improving the emergent viscoelastic properties of these hydrogels, with both Fmoc-3F-Phe and Fmoc-F5-Phe hydrogels showing enhanced stability relative to Fmoc-Phe hydrogels.[7,40,56,60,67-68,74] We formulated these gels under two comparative gelation conditions. First, we used a solvent switch method (see Materials and Methods for details) in which concentrated solutions of each gelator in DMSO were diluted into water to a final gelator concentration of 5 mM (2% DMSO/water, *v/v*). After dilution, the gelators initially form insoluble suspensions. In the case of Fmoc-3F-Phe and Fmoc-F5-Phe, these suspensions rapidly (< 5 minutes) form optically transparent self-supporting hydrogels. In contrast, Fmoc-Phe fails to form a self-supporting hydrogel under these conditions; the gelator instead precipitates from solution over time. We were limited to studying hydrogels of 5 mM gelator under these conditions since higher concentrations of gelator either failed to form hydrogels or formed optically opaque gels. Second, we formulated the hydrogels by a pH adjustment method (see Materials and Methods for details).[42,57,65,71-72] The gelators were dissolved in basic water which solubilizes the gelators by deprotonation of the C-terminal carboxylic acid. Gelation was initiated by addition of glucono-δ-lactone (GdL), which slowly hydrolyzes under basic conditions to generate a carboxylic acid that gradually modifies the pH from basic to more acidic. Gelation occurs as the solution neutralizes

as a function of GdL hydrolysis. Gelation under these conditions typically requires approximately 24 hours, but Fmoc-3F-Phe and Fmoc-F5-Phe form optically transparent hydrogels at varying concentrations of gelator. The adjustment of pH by the direct addition of acid results in rapid rate of gelation that provides low quality gels of highly variable viscoelasticity.[64-65] Adjustment of pH by the gradual hydrolysis of GdL has been shown to result in much more uniform hydrogels, so this method was adopted for these studies.[64-65] We analyzed gels of 5 mM (low concentration) and 15 mM (high concentration) for all three gelators.

We first assessed the thixotropic self-healing behavior of these hydrogels at the bulk scale. When these supramolecular hydrogels are mechanically agitated (for example, by vigorous mixing by vortex), the applied shear forces disrupt the hydrogel network, resulting in gel-sol transitions as a function of the weakened network. In some cases, the hydrogel network is reestablished over time and the hydrogels are reformed in an illustration of self-healing behavior. In order to assess this self-healing property for each hydrogel formed by either the solvent switch or pH adjustment methods, hydrogels were formed in vials and mechanically disrupted by mixing by vortex. After allowing time for recovery, the disrupted hydrogels were assessed by inversion to determine the self-healing capabilities of each system. Fmoc-3F-Phe and Fmoc-F5-Phe hydrogels which had been formed by solvent exchange from DMSO showed precipitation of the network fibrils immediately upon mechanical disruption (see **Figures 2A** and **2B** for a representative example of Fmoc-F5-Phe) as has been previously reported.[40,56,60] Precipitation of the fibril network was accompanied by a loss in hydrogel integrity. These gels failed to rapidly self-heal over a period of two minutes, although weak heterogenous hydrogels were sometimes observed several hours after mechanical disruption. Fmoc-3F-Phe and Fmoc-F5-Phe hydrogels formulated under solvent switch conditions also displayed poor stability as a function of time,

with evidence of precipitation in non-agitated hydrogels beginning within 24 hours. Again, Fmoc-Phe fails to form hydrogels altogether using this DMSO solvent switch formulation method.

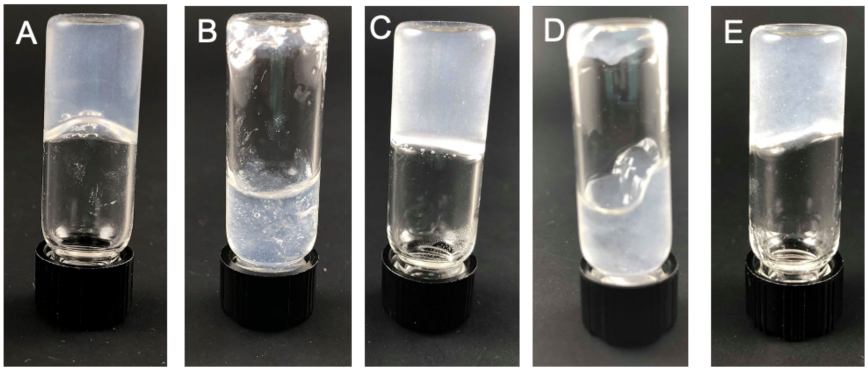


Figure 2. Representative images of hydrogels subjected to mechanical agitation to assess bulk self-healing properties. A) Fmoc-F5-Phe hydrogel (5 mM) formed by the DMSO solvent switch method before mechanical agitation. B) Fmoc-F5-Phe (5 mM) formed by the DMSO solvent switch method two minutes after mechanical disruption. The fibril network has partially precipitated and the hydrogel has failed to reform. C) Fmoc-F5-Phe hydrogel (5 mM) formed by the GdL pH adjustment method prior mechanical disruption. D) Fmoc-F5-Phe hydrogel (5 mM) formed by the GdL pH adjustment method immediately after mechanical disruption. E) Fmoc-F5-Phe hydrogel (5 mM) formed by the GdL pH adjustment method, 30 seconds after mechanical disruption.

In contrast, hydrogels of Fmoc-3F-Phe and Fmoc-F5-Phe formulated by the GdL pH adjustment method uniformly exhibited bulk self-healing properties after mechanical agitation. Several advantages of this formulation method for Fmoc-Phe-derived supramolecular hydrogels are evident in this analysis. First, while Fmoc-Phe itself fails to form hydrogels altogether by the DMSO solvent switch method, it readily forms hydrogels by pH adjustment with GdL, though under different conditions that studied in this paper.[65,71] Second Fmoc-3F-Phe and Fmoc-F5-Phe were found to form hydrogels at a range of concentrations (5 mM and 15 mM hydrogels were assessed herein), while hydrogels formed using the DMSO solvent switch strategy were

observed to form only at concentrations up to 5 mM (precipitation of gelator was observed at higher concentrations). It should be noted that gelation under DMSO solvent switch conditions occurs within 3–5 minutes, while gelation by GdL pH adjustment requires 12–24 hours. Finally, the gels formed by GdL pH adjustment did not immediately precipitate upon mechanical disruption. Instead, the gels were found to undergo temporary shear-thinning (see **Figures 2C** and **2D** for representative images of Fmoc-F5-Phe hydrogels (5 mM) before and immediately after mechanical agitation). These shear-thinned hydrogels rapidly reformed a hydrogel network: within 30 seconds after agitation self-supporting hydrogels that were stable to vial inversion were observed for all three hydrogels at both low (5 mM) and high (15 mM) concentrations (see **Figure 2E** for a representative image of an Fmoc-F5-Phe (5 mM) hydrogel).

Thixotropic viscoelastic rheological properties of hydrogels formed by solvent exchange from DMSO

Next, we characterized the emergent thixotropic properties of these hydrogels by oscillatory rheology. We have previously reported that gels of Fmoc-3F-Phe and Fmoc-F5-Phe formed by dilution from DMSO into water are mechanically unstable and are not self-healing.[40,56,60] Specifically, we have previously observed that these hydrogels undergo precipitation of the gel network after mechanical agitation or over time and that these precipitated fibrils fail to reestablish a network. These observations at the bulk scale were confirmed in this study as described in the previous section. However, further investigation by oscillatory rheology suggests that these macroscopic bulk observations may be somewhat misleading. When shear recovery of these gels is monitored for only a few minutes at the bulk scale, it does appear as if there is no self-healing behavior. When recovery is monitored over

longer periods of hours, some shear recovery is observed at the bulk scale in cases where a lesser degree of network precipitation has occurred. Quantitative characterization of the emergent viscoelastic character of these gels will provide further insight into the self-healing behavior of these materials.

Prior to analyzing the thixotropic rheological properties of these gels we first characterized the fundamental viscoelastic character of each material to ensure it was similar to previously reported data.[40,56,60,73-74] Specifically, we first performed strain sweep analyses of hydrogels of Fmoc-3F-Phe and Fmoc-5F-Phe formed by dilution from DMSO into water (5 mM gelator, 2% DMSO/water, *v/v*; see Materials and Methods for experimental details). The strain sweep analyses (Figures S1 and S4) were used to determine the linear viscoelastic region for each material in order to identify an appropriate strain value for subsequent dynamic frequency sweep and time sweep experiments. Dynamic frequency sweep experiments are used to characterize the viscoelastic storage and loss moduli (G′ and G″, respectively) of each hydrogel (see Supplementary Information, Figures S8 and S10 for this data). The hydrogels were deposited onto the rheometer plate immediately after dilution of the gelators from DMSO into water and gelation was allowed to proceed on the plate for 60 minutes. Once the gels formed, frequency sweeps were conducted at 1% strain from 0.1–100 rad s^{-1}. Interestingly, the storage moduli of these frequency sweeps exhibited slight non-linearity as increasing frequency, consistent with shear-thickening/thinning behavior (Figure S8 and S10). The departure from non-linearity was subtle, however.

Next, we characterized the thixotropic properties the Fmoc-3F-Phe and Fmoc-F5-Phe hydrogels using dynamic time sweep experiments. Dynamic time sweeps were performed by depositing the suspension that results immediately after dilution of the gelator from DMSO into

water on the rheometer plate and observing gelation at low strain (0.2%) for 85 minutes (**Figure 3**). Over this initial gelation period, the storage modulus (G′) values increased from 3128–56,871 Pa for Fmoc-3F-Phe hydrogels and from 1300–1750 Pa for Fmoc-F5-Phe hydrogels. The storage modulus for Fmoc-3F-Phe hydrogels is significantly higher than reported previously,[41] although hydrogel viscoelasticity of materials formed by dilution from DMSO into water can be highly variable, and in this study, these values accurately reflect the observed viscoelasticity of these hydrogels across the replicate experiments (see Supplementary Information Figure S12). The G′ values exceeded the loss modulus (G″) values, and the gap between them increases through the duration of the initial gelation step. This initial gelation phase was followed by a 2.5-minute period at high strain (100%), then an hour at 0.2% strain, then 5 minutes at 100% strain, and followed by a final hour at 0.2% strain. During the short strain period, the storage and loss moduli invert with G″ exceeding G′, consistent with the shear-thinning conversion of the hydrogel to a fluid. In the recovery period following this strain, the G′ values again increased from 2671–53756 Pa and 889–1544 Pa for Fmoc-3F-Phe and Fmoc-F5-Phe, respectively, consistent with self-healing shear recovery. These values are slightly lower than the initial formation values. Interestingly, Fmoc-F5-Phe exhibited a much larger difference between the G′ and G″ values in this recovery period than it had under initial formation, suggesting that it may have formed a more stable gel network after initial disruption. During the second strain period, the values of G′ and G″ once again invert as the material shear thins in response to the applied strain. The final recovery period provided G′ values increased from 961–44628 Pa and 1072–1718 Pa for Fmoc-3F-Phe and Fmoc-F5-Phe, respectively. This data indicates that contrary to observations that these hydrogels at the bulk scale do not display shear

thinning/shearing recovery properties, these materials do exhibit slow self-healing shear recovery behavior over longer recovery periods.

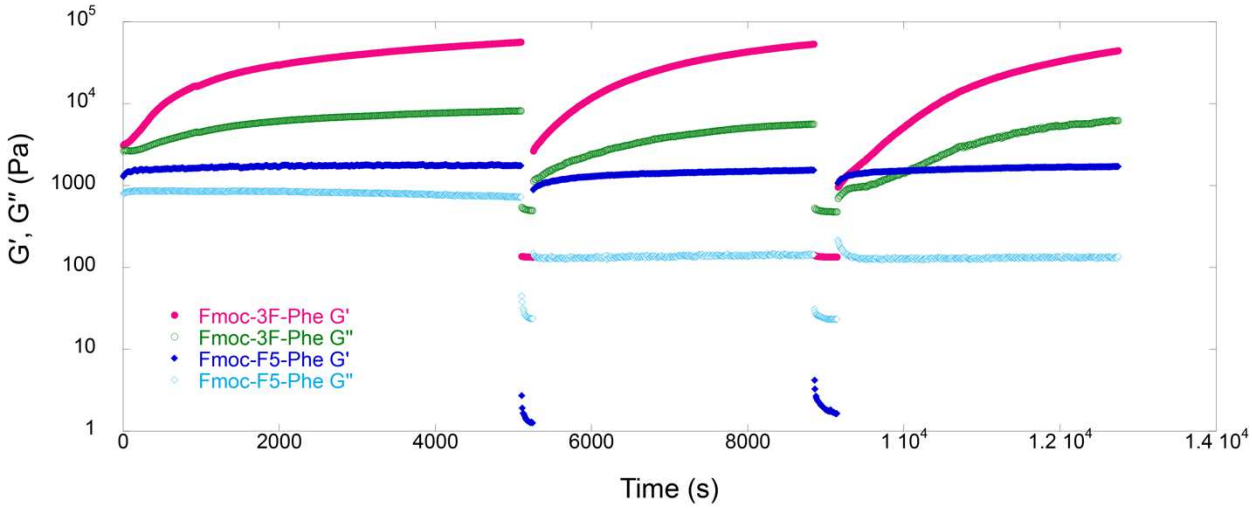


Figure 3. Dynamic time sweep of DMSO method gels which were assembled on plate. Data is an average of three runs. Fmoc-3F-Phe G′ (pink), Fmoc-3F-Phe G′′ (green), Fmoc-F5-Phe G′ (dark blue), and Fmoc-F5-Phe G′′ (light blue).

We also attempted to conduct these rheological experiments under conditions in which the hydrogels were allowed to form prior to deposition of the gels on the rheometer plate (as opposed to allowing the gels for form *in situ*). These experiments proved to be problematic. It was found that the rheological shear-recovery data was inconsistent with the data reported in **Figure 3** when gelation occurred on the rheometer under strain (see Supplementary Information Figures S13–S14 for data on hydrogels formed prior to deposition on the rheometer stage). In contrast to the gradual recovery which is reflective of the hydrogel behavior on the bulk scale, these data show immediate recovery. Upon close examination it was observed that hydrogels of Fmoc-3F-Phe and Fmoc-F5-Phe that were preformed and then deposited on the rheometer stage underwent syneresis over the course of the rheological analysis. The gels were found to contract, and the liquid separated from the gels; this was often accompanied by precipitation of the fibril network. These findings are consistent with the observed instability of these hydrogels

formulated under solvent switch conditions at the bulk scale, where the network was found to precipitate upon mechanical agitation. It is interesting to note that the rheological properties of these supramolecular hydrogels differ when gelation occurs under strain versus under quiescent conditions even when the solvent gelation trigger conditions are identical.

Thixotropic viscoelastic rheological properties of hydrogels formed by pH adjustment via GdL hydrolysis

Next, we investigated the comparative rheological viscoelastic properties of hydrogels formulated by pH modification through GdL hydrolysis (see Materials and Methods for experimental details). Hydrogels formed by dilution of the gelator into water from DMSO are limited to concentrations of approximately 5 mM, since precipitation was often observed at higher concentrations. In contrast, hydrogels formed by the gradual adjustment of pH from basic to neutral (or acidic) could be formulated at significantly higher concentrations. Thus, we conducted rheological analyses at low (5 mM, identical to the DMSO dilution hydrogel concentrations) and high (15 mM) concentrations. Unlike the DMSO gels, which form within minutes, hydrogelation triggered by hydrolysis of GdL occurs much more slowly, typically requiring up to 24 hours. Thus, rheological analyses could not be conducted by forming the hydrogels in situ on the rheometer stage. Instead, these hydrogels were pre-formed in tubes and transferred to the rheometer stage for analysis. Interestingly, the GdL gels proved to be completely stable to this manipulation whereas the DMSO dilution gels underwent significant syneresis upon attempts to transfer them to the rheometer stage. This observation is consistent with the GdL hydrogels having significantly enhanced thixotropic shear-responsive properties. Hydrogels made from Fmoc-3F-Phe show initial G′ values of 367–413 Pa and 4408–5613 Pa for

5 mM and 15 mM gels, respectively. Following the first short strain period, the G′ values were 425–479 Pa and 5856–6717 Pa for 5 mM and 15 mM gels, respectively. The final recovery period following the longer strain period showed G′ values of 465–536 Pa and 5785–7360 Pa for 5 mM and 15 mM gels, respectively. Hydrogels made from Fmoc-F5-Phe show initial G′ values of 383–438 Pa and 5519–6523 Pa for 5 mM and 15 mM gels, respectively. Following the first short strain period, the G′ values were 385–438 Pa and 6171–6875 Pa for 5 mM and 15 mM gels, respectively. The final recovery period following the longer strain period showed G′ values of 425–482 Pa and 6563–7126 Pa for 5 mM and 15 mM gels, respectively. The strain sweep analyses (Figures S2–S3 and S5–S6) were used to determine the linear viscoelastic region for each material in order to identify an appropriate strain value for subsequent dynamic frequency sweep and time sweep experiments. Dynamic frequency sweep experiments are used to characterize the viscoelastic storage and loss moduli (G′ and G″, respectively) of each hydrogel (see Supplementary Information, Figures S9 and S11 for this data). As with the hydrogels formulated by dilution from DMSO, these hydrogels also showed slight increases in storage moduli as frequencies increased, consistent with subtle shear-thickening behavior.

The thixotropic properties of Fmoc-3F-Phe and Fmoc-F5-Phe hydrogels formed by pH adjustment were then characterized using dynamic time sweep experiments. Hydrogels were transferred to the rheometer stage and dynamic time sweep experiments were conducted a constant frequency of 1 Hz (6.28 rad s^{-1}) with a strain of 0.2% for five minutes, followed by a 2.5-minute period of 100% strain, then 0.2% strain for five minutes, then 100% strain for five minutes, and finally 0.2% strain for five minutes. These time sweep experiments clearly demonstrate the rapid shear thinning and shear recovery of both high and low concentration of each of the hydrogels formed by graduate pH adjustment (**Figure 4**). During the short high

strain periods, the storage and loss moduli rapidly invert with G″ exceeding G′, indicating shear-thinning of the hydrogels. Once the high strain was reduced, shear recovery as evidenced by reversion of G′ and G″ to the initially observed values occurred almost instantaneously for both high and low concentration hydrogels. This shear recovery behavior was observed after every repeated cycle of low to high to low strain, indicating that these hydrogels have excellent thixotropic self-healing properties. These data are especially striking when compared to the weak shear recovery observed for these hydrogels when formulated by dilution from DMSO into water.

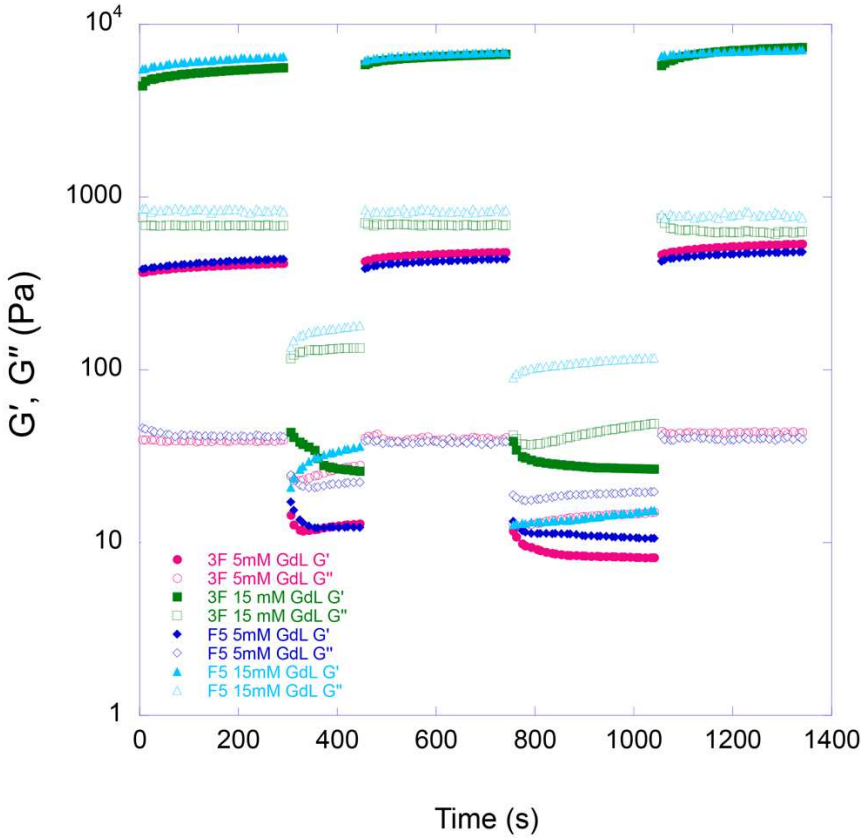


Figure 4. Dynamic time sweep analysis of Fmoc-3F-Phe, and Fmoc-F5-Phe hydrogels formulated by pH adjustment by GdL hydrolysis. Data is an average of three runs. G′ values are represented by filled circles and G″ values are represented by open circles. Fmoc-3F-Phe 5 mM gels (pink), Fmoc-3F-Phe 15 mM gels (green), Fmoc-F5-Phe 5 mM gels (dark blue), and Fmoc-F5-Phe gels (light blue).

Morphology of the constituent fibrils

Transmission electron microscopy was used to image the fibrils that comprise the hydrogel networks in each of these conditions (**Figure 5**). This was done in order to determine if any differences in fibril morphology might be responsible for the dramatic differences in the observed thixotropic properties of these differing gelation methods. We found that there were not significant morphological differences which would account for the drastic changes in behavior exhibited by hydrogels formed by different methods. This indicates that the difference is likely less related to packing and more related to the network of the fibrils themselves. Fibrils of Fmoc-3F-Phe formed by the solvent switch method were 22 ± 3 nm wide (Figure 5A). The fibrils of Fmoc-3F-Phe formed by the pH adjustment method were 11 ± 2 nm wide and 19 ± 5 nm wide for 5 mM and 15 mM, respectively (Figure 5B and 5C). Fibrils formed from Fmoc-F5-Phe using the solvent switch method had a width of 15 ± 2 nm (Figure 5D). The fibrils of Fmoc-F5-Phe formed by pH adjustment had widths of 24 ± 8 nm and 16 ± 3 nm for 5 mM and 15 mM, respectively.

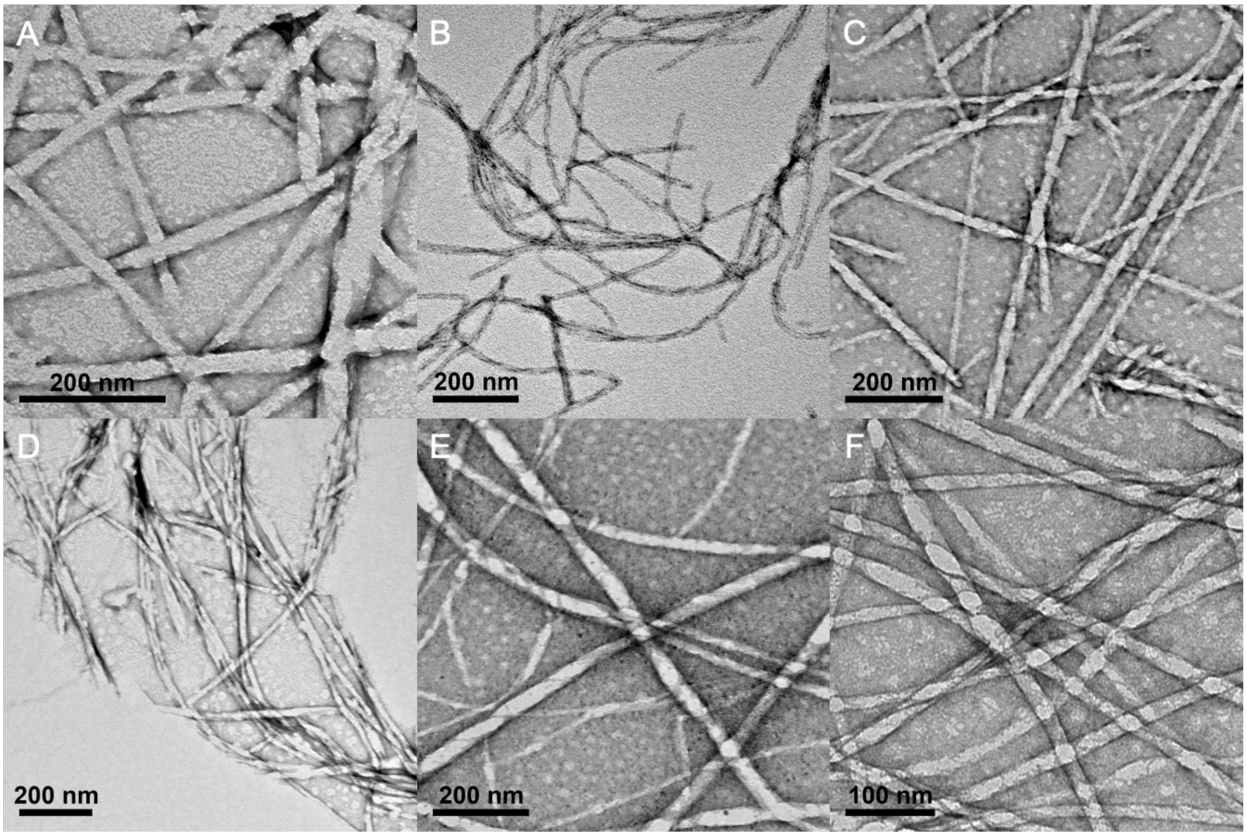
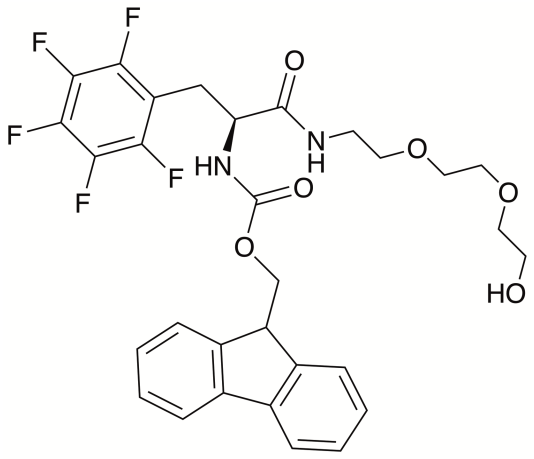


Figure 5. Transmission electron micrographs of Fmoc-Phe derived hydrogels. A) Fmoc-3F-Phe 5 mM solvent switch method; B) Fmoc-3F-Phe 5 mM pH adjustment method; C) Fmoc-3F-Phe 15 mM pH adjustment method; D) Fmoc-F5-Phe 5 mM solvent switch method; E) Fmoc-F5-Phe 5 mM pH adjustment method; F) Fmoc-F5-Phe 15 mM pH adjustment method.

Discussion

Thixotropic shear recovery properties are critical for hydrogels intended for biomedical applications. This is especially true for those materials intended for delivery to a biological system by injection for applications in drug delivery and regenerative medicine. To date, there are many examples of supramolecular hydrogels derived from self-assembled peptides that exhibit these thixotropic properties.[75-77] The design of inexpensive LMW supramolecular

hydrogels that possess shear recovery properties has been more challenging.[11,51-53] For example, in our initial reports of hydrogels formed by self-assembling halogenated Fmoc-Phe derivatives, we observed that the gels did not exhibit robust thixotropic shear recovery. This rendered hydrogels of these derivatives unstable upon mechanical agitation which most often resulted in precipitation of the hydrogel network. In these early reports, the singular gelation method was dilution of the gelator from DMSO into water. In an effort to engineer supramolecular Fmoc-F5-Phe hydrogels that possessed thixotropic properties that would impart stability to mechanical agitation, we formulated multicomponent hydrogels composed of Fmoc-F5-Phe mixed with a pegylated derivative, Fmoc-F5-Phe-PEG (**Figure 6**).[56] When Fmoc-F5-Phe and Fmoc-F5-Phe-PEG were mixed at varying ratios in DMSO and diluted into water the hydrogels that formed exhibited the desired thixotropic shear recovery properties. This strategy was later found to impart the same shear recovery characteristics to hydrogels of other Fmoc-Phe derivatives that were formulated under these solvent switch conditions.[78]



Fmoc-F5-Phe-PEG

Figure 6. Chemical structure of Fmoc-F5-Phe-PEG.

In several recent reports, Adams and coworkers have articulated the significant effect that gelation conditions can exert on the emergent properties of supramolecular hydrogels.[57-59,62,79] They observed that the values of the viscoelastic properties of supramolecular hydrogels formed from the N-terminal Fmoc-modified diphenylalanine peptide (Fmoc-FF) varied by over 4 orders of magnitude.[57] In response to this dramatic variability, they sought to understand the effect of gelation conditions on the emergent viscoelasticity of the resulting hydrogels and found that differences in solvent conditions, including pH, introduced significant alterations in these properties. Our comparative analysis of Fmoc-Phe derived supramolecular hydrogels herein is clearly consistent with their findings. Our analysis is specifically focused on thixotropic shear recovery properties, and the emergent thixotropic properties of Fmoc-Phe-derived supramolecular hydrogels are dramatically impacted by gelation method. Fmoc-3F-Phe and Fmoc-F5-Phe hydrogels formulated by dilution from DMSO into water were poorly shear responsive at bulk scales and exhibited only slow shear recovery under specific rheological conditions (that is, when hydrogels were allowed to form *in situ* on the rheometer stage under strain). In contrast, hydrogels of these same agents that were formulated by dissolution in water under basic conditions followed by gradual reduction in pH by GdL hydrolysis had excellent thixotropic properties, showing rapid shear recovery at both bulk scales and rheologically. Adams and coworkers concluded that the final pH of the gels were responsible for the differing properties of Fmoc-FF hydrogels,[57] and it is possible that this same effect is at play with Fmoc-3F-Phe and Fmoc-F5-Phe hydrogels. Hydrogels of Fmoc-3F-Phe and Fmoc-F5-Phe formed by DMSO solvent switch have pH values between 3 and 5,[67] and those formed by GdL hydrolysis have been found have pH values of 4–7, depending on both the concentration of gelator and concentration of GdL.[74]

We also considered the possibility that additives may play a role in the emergent gelation properties. Some additives have been found to impact the viscoelastic properties of Fmoc-FF hydrogels.[57-58] In this study, hydrogels formulated by the hydrolysis of GdL possess at least one molar equivalent of the hydrolyzed by-product, gluconic acid. Adams and coworkers have previously investigated whether or not GdL or its hydrolysis product interfere with self-assembly and hydrogelation using saturation transfer difference NMR and have concluded that it does not.[80-81] In order to test the possible effect of gluconic acid on hydrogel shear recovery properties we added one molar equivalent of D-glucitol (the reduced alcohol form of gluconic acid was used to eliminate issues with pH modification from the acid) was added to Fmoc-F5-Phe in DMSO and diluted into water at 5 mM gelator. When these samples were mechanically agitated by vortex mixing, the hydrogel network was disrupted and failed to recover as was observed in these hydrogels without D-glucitol (**Figure 2**). Thus, the presence of this additive had no effect on thixotropic shear recovery properties under these gelation conditions, with the outcomes indistinguishable from the gelator alone in this bulk stability test.

The mechanism of self-assembly can also dramatically impact the emergent viscoelasticity of supramolecular hydrogels.[58] This may be especially true of the gelation methods we discuss herein. We have observed dramatic differences in rate of assembly and gelation under the two conditions. Hydrogels formulated using the DMSO solvent switch method typically form very rapidly (< 3 minutes) whereas the hydrogels formed by pH reduction through GdL hydrolysis require 12–24 hours to fully mature. The dramatically accelerated rate of assembly of the Fmoc-Phe derivatives under DMSO dilution conditions may result in a more sporadically cross-linked fibril network that exists in a kinetically trapped state. This kinetic state may provide a more fragile gel network that cannot undergo shear recovery because the fibrils

that form are solvolytically unstable, resulting in precipitation and/or syneresis upon the application of shear forces, which represents a more thermodynamically stable state than the kinetic hydrogel. The network may be less ordered and more sporadic and idiosyncratic in terms of entanglement. In contrast, the hydrogels formed by gradual adjustment of pH assemble much more slowly. This slow rate of assembly may result in a hydrogel network that is significantly more energetically stable, eliminating complications with precipitation since that state no longer represents a more thermodynamically favorable condition. We have no data that suggests that the packing mode of the self-assembled derivatives has been altered, and thus the most likely explanation for the observed differences in thixotropic properties is at the level of how the self-assembled fibrils are entangled into a network. Perhaps the slower assembly rate provides a more ordered and efficiently entangled network in which more contacts are made between fibrils, providing a more stable energetic state.

It is significant that formulation method can be used to tune the properties of Fmoc-Phe derived hydrogels. A single gelation method does not, in the case of these materials, represent the "ideal" manner of formulation, since different applications have different requirements. For example, injectable hydrogels for drug delivery depend on thixotropic shear recovery properties to enable delivery of the hydrogel by convenient injection.[73-74] Drug delivery applications do not require that the carrier gel form rapidly, making the use of the GdL formulation method appropriate for the preparation of materials for this purpose. In contrast, *in vitro* tissue engineering applications often require a gel network that can suspend cells throughout the three-dimensional network. This demands a formulation method that facilitates rapid hydrogelation in minutes in order to prevent cell sedimentation during the gelation process, which would provide a heterogenous distribution of cells throughout the network. Thus, the GdL pH adjustment

method is obviously problematic for this type of application due to the slow rate of gelation as well as the need to place cells in a very basic environment for a time period of hours. To date, no single gel method is yet universally ideal for all biomedical applications, but a greater understanding of how formulation methods correlate to emergent viscoelastic properties is a significant step toward optimizing these materials for a broad range of uses.

Conclusion

LMW supramolecular hydrogels are promising next-generation materials for a broad range of biomedical applications. They are especially attractive as inexpensive alternatives to functionally ideal but cost prohibitive peptide-based materials.[23] The limited chemical space available for modification in LMW gelators creates significant constraints on strategies to modify and optimize the emergent properties of these hydrogel materials. These constraints have impeded the more widespread adoption of LMW gelators for *in vivo* biological applications. The work reported herein provides significant insight into a strategy other than chemical modification of the gelator to tune the viscoelastic thixotropic properties of LMW hydrogels. Specifically, the formulation conditions can be used to dramatically influence these emergent properties. In this work, we have compared two methods for the preparation of Fmoc-Phe-derived LMW supramolecular hydrogels and found that in one case (solvent switch) the resulting hydrogels are poorly shear-responsive and in the other case (pH adjustment) the resulting materials are ideally shear-responsive. This confirmation that varying gelation method can be used as a primary strategy to alter the desired properties of LMW hydrogels is a significant step toward overcoming existing barriers in the practical adoption of these promising materials. Future work

that correlates the fundamental physicochemical principles that underlie these empirical studies will further reduce these barriers.

Conflict of Interest

There are no conflicts to declare.

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Supplementary Information

The Supplementary Information (SI) is available free of charge. SI includes additional rheological data.

References

1. Ghosh, G.; Dey, P.; Ghosh, S. Controlled supramolecular polymerization of pi-systems. *Chem. Commun.* **2020,** *56*, 6757-6769.
2. Hoque, J.; Sangaj, N.; Varghese, S. Stimuli-Responsive Supramolecular Hydrogels and Their Applications in Regenerative Medicine. *Macromol. Biosci.* **2019,** *19*, e1800259.
3. Du, X.; Zhou, J.; Shi, J.; Xu, B. Supramolecular Hydrogelators and Hydrogels: From Soft Matter to Molecular Biomaterials. *Chem. Rev.* **2015,** *115*, 13165-13307.

4. Hanabusa, K.; Suzuki, M. Physical Gelation by Low-Molecular-Weight Compounds and Development of Gelators. *Bull. Chem. Soc. Jpn.* **2016,** *89*, 174-182.
5. Strandman, S.; Zhu, X. X. Self-Healing Supramolecular Hydrogels Based on Reversible Physical Interactions. *Gels* **2016,** *2*.
6. Savyasachi, A. J.; Kotova, O.; Shanmugaraju, S.; Bradberry, S. J.; Ó'Máille, G. M.; Gunnlaugsson, T. Supramolecular Chemistry: A Toolkit for Soft Functional Materials and Organic Particles. *Chem* **2017,** *3*, 764-811.
7. Liyanage, W.; Nilsson, B. L. Substituent Effects on the Self-Assembly/Coassembly and Hydrogelation of Phenylalanine Derivatives. *Langmuir* **2016,** *32*, 787-799.
8. Guo, J. L.; Kim, Y. S.; Mikos, A. G. Biomacromolecules for Tissue Engineering: Emerging Biomimetic Strategies. *Biomacromolecules* **2019,** *20*, 2904-2912.
9. Samadian, H.; Maleki, H.; Fathollahi, A.; Salehi, M.; Gholizadeh, S.; Derakhshankhah, H.; Allahyari, Z.; Jaymand, M. Naturally occurring biological macromolecules-based hydrogels: Potential biomaterials for peripheral nerve regeneration. *Int. J. Biol. Macromol.* **2020,** *154*, 795-817.
10. Koutsopoulos, S. Self-assembling peptide nanofiber hydrogels in tissue engineering and regenerative medicine: Progress, design guidelines, and applications. *J. Biomed. Mater. Res. A* **2016,** *104*, 1002-1016.
11. Dastidar, P. Supramolecular gelling agents: can they be designed? *Chem. Soc. Rev.* **2008,** *37*, 2699-2715.
12. Liu, C.; Zhang, Q.; Zhu, S.; Liu, H.; Chen, J. Preparation and applications of peptide-based injectable hydrogels. *RSC Adv.* **2019,** *9*, 28299-28311.
13. Guvendiren, M.; Lu, H. D.; Burdick, J. A. Shear-thinning hydrogels for biomedical applications. *Soft Matter* **2012,** *8*, 260-272.
14. Dawn, A.; Kumari, H. Low Molecular Weight Supramolecular Gels Under Shear: Rheology as the Tool for Elucidating Structure-Function Correlation. *Chem. Eur. J.* **2018,** *24*, 762-776.
15. Yan, C.; Altunbas, A.; Yucel, T.; Nagarkar, R. P.; Schneider, J. P.; Pochan, D. J. Injectable solid hydrogel: mechanism of shear-thinning and immediate recovery of injectable beta-hairpin peptide hydrogels. *Soft Matter* **2010,** *6*, 5143-5156.
16. Taylor, D. L.; In Het Panhuis, M. Self-Healing Hydrogels. *Adv. Mater.* **2016,** *28*, 9060-9093.
17. Bowerman, C. J.; Nilsson, B. L. Self-assembly of amphipathic beta-sheet peptides: insights and applications. *Biopolymers* **2012,** *98*, 169-84.
18. Yu, Z.; Cai, Z.; Chen, Q.; Liu, M.; Ye, L.; Ren, J.; Liao, W.; Liu, S. Engineering beta-sheet peptide assemblies for biomedical applications. *Biomater. Sci.* **2016,** *4*, 365-374.
19. Dehsorkhi, A.; Castelletto, V.; Hamley, I. W. Self-assembling amphiphilic peptides. *J. Pept. Sci.* **2014,** *20*, 453-67.
20. Seow, W. Y.; Hauser, C. A. E. Short to ultrashort peptide hydrogels for biomedical uses. *Materials Today* **2014,** *17*, 381-388.
21. Amit, M.; Yuran, S.; Gazit, E.; Reches, M.; Ashkenasy, N. Tailor-Made Functional Peptide Self-Assembling Nanostructures. *Adv. Mater.* **2018,** *30*, e1707083.
22. Mayr, J.; Saldias, C.; Diaz Diaz, D. Release of small bioactive molecules from physical gels. *Chem. Soc. Rev.* **2018,** *47*, 1484-1515.
23. Sis, M. J.; Webber, M. J. Drug Delivery with Designed Peptide Assemblies. *Trends Pharmacol. Sci.* **2019,** *40*, 747-762.

24. Orbach, R.; Adler-Abramovich, L.; Zigerson, S.; Mironi-Harpaz, I.; Seliktar, D.; Gazit, E. Self-assembled Fmoc-peptides as a platform for the formation of nanostructures and hydrogels. *Biomacromolecules* **2009,** *10*, 2646-2651.
25. Wang, Y.; Zhang, Z.; Xu, L.; Li, X.; Chen, H. Hydrogels of halogenated Fmoc-short peptides for potential application in tissue engineering. *Colloids Surf B Biointerfaces* **2013,** *104*, 163-8.
26. Hanabusa, K.; Suzuki, M. Development of low-molecular-weight gelators and polymer-based gelators. *Polym. J. (Tokyo, Jpn.)* **2014,** *46*, 776-782.
27. Raeburn, J.; Zamith Cardoso, A.; Adams, D. J. The importance of the self-assembly process to control mechanical properties of low molecular weight hydrogels. *Chem Soc Rev* **2013,** *42*, 5143-56.
28. Li, R.; Horgan, C. C.; Long, B.; Rodriguez, A. L.; Mather, L.; Barrow, C. J.; Nisbet, D. R.; Williams, R. J. Tuning the mechanical and morphological properties of self-assembled peptide hydrogels via control over the gelation mechanism through regulation of ionic strength and the rate of pH change. *RSC Adv.* **2015,** *5*, 301-307.
29. Kurbasic, M.; Romano, C. D.; Garcia, A. M.; Kralj, S.; Marchesan, S. Assembly of a Tripeptide and Anti-Inflammatory Drugs into Supramolecular Hydrogels for Sustained Release. *Gels* **2017,** *3*.
30. Nanda, J.; Biswas, A.; Banerjee, A. Single amino acid based thixotropic hydrogel formation and pH-dependent morphological change of gel nanofibers. *Soft Matter* **2013,** *9*.
31. Marchesan, S.; Vargiu, A. V.; Styan, K. E. The Phe-Phe Motif for Peptide Self-Assembly in Nanomedicine. *Molecules* **2015,** *20*, 19775-19788.
32. Eckes, K. M.; Mu, X.; Ruehle, M. A.; Ren, P.; Suggs, L. J. beta sheets not required: combined experimental and computational studies of self-assembly and gelation of the ester-containing analogue of an Fmoc-dipeptide hydrogelator. *Langmuir* **2014,** *30*, 5287-5296.
33. Fichman, G.; Guterman, T.; Adler-Abramovich, L.; Gazit, E. Synergetic functional properties of two-component single amino acid-based hydrogels. *CrystEngComm* **2015,** *17*, 8105-8112.
34. Liyanage, W.; Vats, K.; Rajbhandary, A.; Benoit, D. S.; Nilsson, B. L. Multicomponent dipeptide hydrogels as extracellular matrix-mimetic scaffolds for cell culture applications. *Chem. Commun.* **2015,** *51*, 11260-11263.
35. Buerkle, L. E.; Rowan, S. J. Supramolecular gels formed from multi-component low molecular weight species. *Chem Soc Rev* **2012,** *41*, 6089-102.
36. Tao, K.; Levin, A.; Adler-Abramovich, L.; Gazit, E. Fmoc-modified amino acids and short peptides: simple bio-inspired building blocks for the fabrication of functional materials. *Chem Soc Rev* **2016,** *45*, 3935-53.
37. Fichman, G.; Gazit, E. Self-assembly of short peptides to form hydrogels: design of building blocks, physical properties and technological applications. *Acta Biomater.* **2014,** *10*, 1671-1682.
38. Adler-Abramovich, L.; Gazit, E. The physical properties of supramolecular peptide assemblies: from building block association to technological applications. *Chem. Soc. Rev.* **2014,** *43*, 6881-6893.
39. Johnson, E. K.; Adams, D. J.; Cameron, P. J. Peptide based low molecular weight gelators. *J. Mater. Chem.* **2011,** *21*, 2024-2027.

40. Ryan, D. M.; Anderson, S. B.; Senguen, F. T.; Youngman, R. E.; Nilsson, B. L. Self-assembly and hydrogelation promoted by F5-phenylalanine. *Soft Matter* **2010,** *6*, 475-479.
41. Ryan, D. M.; Anderson, S. B.; Nilsson, B. L. The influence of side-chain halogenation on the self-assembly and hydrogelation of Fmoc-phenylalanine derivatives. *Soft Matter* **2010,** *6*.
42. Draper, E. R.; Morris, K. L.; Little, M. A.; Raeburn, J.; Colquhoun, C.; Cross, E. R.; McDonald, T. O.; Serpell, L. C.; Adams, D. J. Hydrogels formed from Fmoc amino acids. *CrystEngComm* **2015,** *17*, 8047-8057.
43. Tao, K.; Yoskovitz, E.; Adler-Abramovich, L.; Gazit, E. Optical property modulation of Fmoc group by pH-dependent self-assembly. *RSC Adv.* **2015,** *5*, 73914-73918.
44. Yang, Z.; Gu, H.; Fu, D.; Gao, P.; Lam, J. K.; Xu, B. Enzymatic Formation of Supramolecular Hydrogels. *Advanced Materials* **2004,** *16*, 1440-1444.
45. Yang, Z.; Liang, G.; Xu, B. Enzymatic hydrogelation of small molecules. *Acc. Chem. Res.* **2008,** *41*, 315-326.
46. Fleming, S.; Debnath, S.; Frederix, P. W.; Tuttle, T.; Ulijn, R. V. Aromatic peptide amphiphiles: significance of the Fmoc moiety. *Chem. Commun.* **2013,** *49*, 10587-10589.
47. Roy, S.; Banerjee, A. Amino acid based smart hydrogel: formation, characterization and fluorescence properties of silver nanoclusters within the hydrogel matrix. *Soft Matter* **2011,** *7*.
48. Das, T.; Haring, M.; Haldar, D.; Diaz Diaz, D. Phenylalanine and derivatives as versatile low-molecular-weight gelators: design, structure and tailored function. *Biomater. Sci.* **2017,** *6*, 38-59.
49. Garcia, A.; Lavendomme, R.; Kralj, S.; Kurbasic, M.; Bellotto, O.; Cringoli, M. C.; Semeraro, S.; Bandiera, A.; De Zorzi, R.; Marchesan, S. Self-assembly of an amino acid derivative into an antimicrobial hydrogel biomaterial. *Chem. Eur. J.* **2019**, 1880-1886.
50. Pizzi, A.; Lascialfari, L.; Demitri, N.; Bertolani, A.; Maiolo, D.; Carretti, E.; Metrangolo, P. Halogen bonding modulates hydrogel formation from Fmoc amino acids. *CrystEngComm* **2017,** *19*, 1870-1874.
51. Adams, D. J. Scrolling for Gels. *Chem* **2017,** *3*, 529-531.
52. Draper, E. R.; Adams, D. J. Low-Molecular-Weight Gels: The State of the Art. *Chem* **2017,** *3*, 390-410.
53. Zhang, Y.; Kuang, Y.; Gao, Y.; Xu, B. Versatile small-molecule motifs for self-assembly in water and the formation of biofunctional supramolecular hydrogels. *Langmuir* **2011,** *27*, 529-537.
54. Singh, V.; Snigdha, K.; Singh, C.; Sinha, N.; Thakur, A. K. Understanding the self-assembly of Fmoc-phenylalanine to hydrogel formation. *Soft Matter* **2015,** *11*, 5353-64.
55. Chivers, P. R. A.; Smith, D. K. Shaping and structuring supramolecular gels. *Nat. Rev. Mater.* **2019,** *4*, 463-478.
56. Ryan, D. M.; Doran, T. M.; Nilsson, B. L. Stabilizing self-assembled Fmoc-F5-Phe hydrogels by co-assembly with PEG-functionalized monomers. *Chem. Commun.* **2011,** *47*, 475-477.
57. Raeburn, J.; Pont, G.; Chen, L.; Cesbron, Y.; Lévy, R.; Adams, D. J. Fmoc-diphenylalanine hydrogels: understanding the variability in reported mechanical properties. *Soft Matter* **2012,** *8*, 1168-1174.

58. Raeburn, J.; Zamith Cardoso, A.; Adams, D. J. The importance of the self-assembly process to control mechanical properties of low molecular weight hydrogels. *Chem. Soc. Rev.* **2013,** *42*, 5143-5156.
59. Colquhoun, C.; Draper, E. R.; Schweins, R.; Marcello, M.; Vadukul, D.; Serpell, L. C.; Adams, D. J. Controlling the network type in self-assembled dipeptide hydrogels. *Soft Matter* **2017,** *13*, 1914-1919.
60. Ryan, D. M.; Anderson, S. B.; Nilsson, B. L. The influence of side-chain halogenation on the self-assembly and hydrogelation of Fmoc-phenylalanine derivatives. *Soft Matter* **2010,** *6*, 3220-3231.
61. Mahler, A.; Reches, M.; Rechter, M.; Cohen, S.; Gazit, E. Rigid, Self-Assembled Hydrogel Composed of a Modified Aromatic Dipeptide. *Advanced Materials* **2006,** *18*, 1365-1370.
62. Raeburn, J.; Mendoza-Cuenca, C.; Cattoz, B. N.; Little, M. A.; Terry, A. E.; Zamith Cardoso, A.; Griffiths, P. C.; Adams, D. J. The effect of solvent choice on the gelation and final hydrogel properties of Fmoc-diphenylalanine. *Soft Matter* **2015,** *11*, 927-935.
63. Liebmann, T.; Rydholm, S.; Akpe, V.; Brismar, H. Self-assembling Fmoc dipeptide hydrogel for in situ 3D cell culturing. *BMC Biotechnol.* **2007,** *7*, 88.
64. Jayawarna, V.; Ali, M.; Jowitt, T. A.; Miller, A. F.; Saiani, A.; Gough, J. E.; Ulijn, R. V. Nanostructured Hydrogels for Three-Dimensional Cell Culture Through Self-Assembly of Fluorenylmethoxycarbonyl–Dipeptides. *Advanced Materials* **2006,** *18*, 611-614.
65. Adams, D. J.; Butler, M. F.; Frith, W. J.; Kirkland, M.; Mullen, L.; Sanderson, P. A new method for maintaining homogeneity during liquid–hydrogel transitions using low molecular weight hydrogelators. *Soft Matter* **2009,** *5*, 1856-1862.
66. Yang, Z.; Liang, G.; Ma, M.; Gao, Y.; Xu, B. Conjugates of naphthalene and dipeptides produce molecular hydrogelators with high efficiency of hydrogelation and superhelical nanofibers. *J. Mater. Chem.* **2007,** *17*, 850-854.
67. Ryan, D. M.; Doran, T. M.; Anderson, S. B.; Nilsson, B. L. Effect of C-terminal modification on the self-assembly and hydrogelation of fluorinated Fmoc-Phe derivatives. *Langmuir* **2011,** *27*, 4029-4039.
68. Ryan, D. M.; Doran, T. M.; Nilsson, B. L. Complementary pi-pi interactions induce multicomponent coassembly into functional fibrils. *Langmuir* **2011,** *27*, 11145-11156.
69. Ryan, D. M.; Nilsson, B. L. Self-assembled amino acids and dipeptides as noncovalent hydrogels for tissue engineering. *Polym. Chem.* **2012,** *3*, 18-33.
70. Abraham, B. L.; Liyanage, W.; Nilsson, B. L. Strategy to Identify Improved N-Terminal Modifications for Supramolecular Phenylalanine-Derived Hydrogelators. *Langmuir* **2019,** *35*, 14939-14948.
71. Sutton, S.; Campbell, N. L.; Cooper, A. I.; Kirkland, M.; Frith, W. J.; Adams, D. J. Controlled release from modified amino acid hydrogels governed by molecular size or network dynamics. *Langmuir* **2009,** *25*, 10285-10291.
72. Adams, D. J.; Mullen, L. M.; Berta, M.; Chen, L.; Frith, W. J. Relationship between molecular structure, gelation behaviour and gel properties of Fmoc-dipeptides. *Soft Matter* **2010,** *6*, 1971-1980.
73. Raymond, D. M.; Abraham, B. L.; Fujita, T.; Watrous, M. J.; Toriki, E. S.; Takano, T.; Nilsson, B. L. Low-Molecular-Weight Supramolecular Hydrogels for Sustained and Localized in Vivo Drug Delivery. *ACS Appl. Bio Mater.* **2019,** *2*, 2116-2124.

74. Abraham, B. L.; Toriki, E. S.; Tucker, N. D. J.; Nilsson, B. L. Electrostatic Interactions Regulate the Release of Small Molecules from Supramolecular Hydrogels. *J. Mater. Chem. B* **2020**, Accepted Manuscript.
75. Zanna, N.; Tomasini, C. Peptide-Based Physical Gels Endowed with Thixotropic Behaviour. *Gels* **2017,** *3*.
76. Zanna, N.; Focaroli, S.; Merlettini, A.; Gentilucci, L.; Teti, G.; Falconi, M.; Tomasini, C. Thixotropic Peptide-Based Physical Hydrogels Applied to Three-Dimensional Cell Culture. *ACS Omega* **2017,** *2*, 2374-2381.
77. Cinar, G.; Ceylan, H.; Urel, M.; Erkal, T. S.; Deniz Tekin, E.; Tekinay, A. B.; Dana, A.; Guler, M. O. Amyloid inspired self-assembled peptide nanofibers. *Biomacromolecules* **2012,** *13*, 3377-3387.
78. Liyanage, W.; Brennessel, W. W.; Nilsson, B. L. Spontaneous Transition of Self-assembled Hydrogel Fibrils into Crystalline Microtubes Enables a Rational Strategy To Stabilize the Hydrogel State. *Langmuir* **2015,** *31*, 9933-9942.
79. Fuentes-Caparros, A. M.; de Paula Gomez-Franco, F.; Dietrich, B.; Wilson, C.; Brasnett, C.; Seddon, A.; Adams, D. J. Annealing multicomponent supramolecular gels. *Nanoscale* **2019,** *11*, 3275-3280.
80. Wallace, M.; Iggo, J. A.; Adams, D. J. Probing the surface chemistry of self-assembled peptide hydrogels using solution-state NMR spectroscopy. *Soft Matter* **2017,** *13*, 1716-1727.
81. Draper, E. R.; Adams, D. J. Controlling the Assembly and Properties of Low-Molecular-Weight Hydrogelators. *Langmuir* **2019,** *35*, 6506-6521.