

Small Peptide-Based Hydrogels: Monitoring Stiffness and Self-Assembly Kinetics by Means of Oscillatory Rheology

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ABSTRACT

The response of selected Fmoc-dipeptides to pH and ionic strength has been studied by oscillatory rheology coupled to microscopy. The gelation rate was proportional to that of protonation of the dipeptide carboxylic group. The ionic strength had also an effect on the gelation kinetics and morphology of the gel obtained.

INTRODUCTION

Hydrogels are an important class of biomaterials because they resemble the extracellular matrix (ECM) of the body and show biocompatibility, thus offering scaffolds for tissue engineering and providing matrices for drug delivery. Recently, stimuli-responsive hydrogelators have received much attention in drug/protein delivery¹. They can release entrapped molecules in response to a stimulus caused by environmental changes such as ionic strength, pH, enzymatic action. In several cases such as tumours, inflammatory tissues, and the phagolysosomes of antigen presenting cells, the drug delivery sites are acidic in nature and a “smart” hydrogel for selective delivery of the drugs would swell or degrade as the local pH lowers. For this purpose, several biocompatible pH-responsive hydrogelators have been synthesized from peptide-based molecules². Several studies have shown that dipeptides

coupled to a fluorenylmethoxycarbonyl (Fmoc) moiety were gelled using a pH trigger, such that the sodium salt of the Fmoc-dipeptide (which is soluble) is converted to the acid form (which gels) by a drop in pH. As protonation occurs, hydrogen-bonds form and the dipeptides assemble from dissolved molecules via micellar structures to crystals and fibrils^{3, 4}. Together with hydrogen bonding other mechanisms that depend on the solution ionic strength, such as the loss of hydrophobic association and the creation of ion-bridges, are likely to influence the self-assembly process⁴⁻⁶. In the present study the effect of the pH and ionic strength on gelation are studied for three model Fmoc-dipeptides. The kinetics of gelation are investigated by means of oscillatory rheometry. This and the measured gel strength are then correlated to its microstructure.

EXPERIMENTAL

Rheology

All chemicals and solvents were purchased from Aldrich or Novabiochem and used as received. Millipore demineralized water (resistivity = 18.2 mΩ) was used throughout. Three model materials chosen were Fmoc-leucine-

glycine (FMOC-LG), FMOC-phenylalanine-glycine (FMOC-FG) and FMOC-phenylalanine-phenylalanine (FMOC-FF). The three FMOC-dipeptides were synthesized and sodium salt of these was made adopting the procedure similar to that described in previous publication⁷, adjusting the exact initial quantity of NaOH rather than the pH to obtain a solution with constant ionic strength prior to gelation. The purity of the materials was confirmed by the absence of unrelated peaks in their NMR signals. Hydrogels were prepared by adding Glucono-delta-Lactone (GdL) after FMOC-dipeptide sodium salts were dissolved in water at a standard concentration of 7.3 mmol/l. The effect of pH variation was studied as a function of the amount of acidifier added. The ionic strength of the solution was varied upon addition of NaCl.

Rheology

Rheology experiments were carried out using a Rheometric Scientific ARES rheometer. A four-bladed vane geometry was used with a diameter of 8.5 mm and length 8.5 mm. The peptide-GdL solution was prepared in a 7 mL Sterilin plastic sample vial, which also served as the cup for the rheological measurements. The walls of the vial were roughened before use in order to avoid slipping of the gel. Once the solution was prepared and the sample vial was mounted on the lower plate of the rheometer, the vane (attached to the upper viscoelastic region of the samples (as tested by stress or strain sweeps after gelation)). It was found that evaporation did not significantly affect results over the timescale of the measurements.

pH timed measurements

A pH302 GLP bench-top pH meter (HANNA instruments) and a Gelplas probe were used to measure the pH. The pH meter was calibrated using pH 4 and pH 7 buffer

solutions, and then rinsed with deionised water. The probe was inserted into the solution and pH readings were recorded every 5 minutes for a minimum of 8 hours.

Cryo-SEM

Low temperature field emission scanning electron microscopy (LT FESEM) was carried out using a JEOL 6301F microscope and Gatan Alto 2500 low temperature equipment.

RESULTS AND DISCUSSION

Effect of PH on the hydrogel formation

A molar excess of acidifier shifts towards the left the protonation reaction of the dipeptide shown in Fig. 1. As a result, more dipeptide molecules will be in neutral acid form. It should also be noted that the preparation of the sodium salt differs from the previously described method as we neutralized the acidic dipeptide using a fixed molar ratio of NaOH to dipeptide⁷. To evaluate the effect of GdL concentration on the gelation, G' and G'' have been recorded in parallel to pH measurements on the same solutions for different excess amounts of added acidifier (GdL). These results are shown in Fig. 2. It is shown in the graphs in Fig. 2 d-f that increasing the amount of GdL in solution leads to a quicker decrease of the pH to lower final values and to less pronounced buffering. By comparing pH and G' curves it can also be noticed that gelation time reduces as GdL excess increases. These data have then been further analyzed to provide further understanding of the gelation mechanism. The time of gel formation has not been taken at the crossover between G' and G'' but arbitrarily at a 90% of the maximum G' value in order to avoid uncertainties due to the initial data noise. As the gelation speed is inversely proportional to this time interval, values of

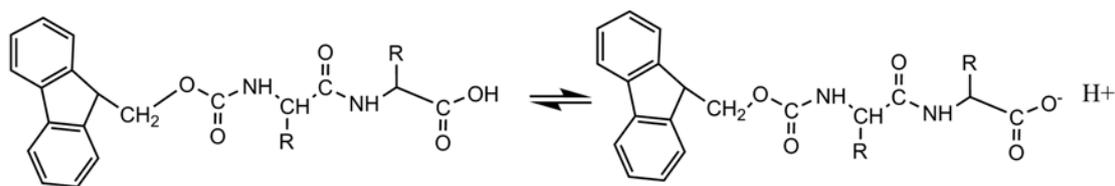


Figure 1. Fmoc-dipeptides protonation reaction.

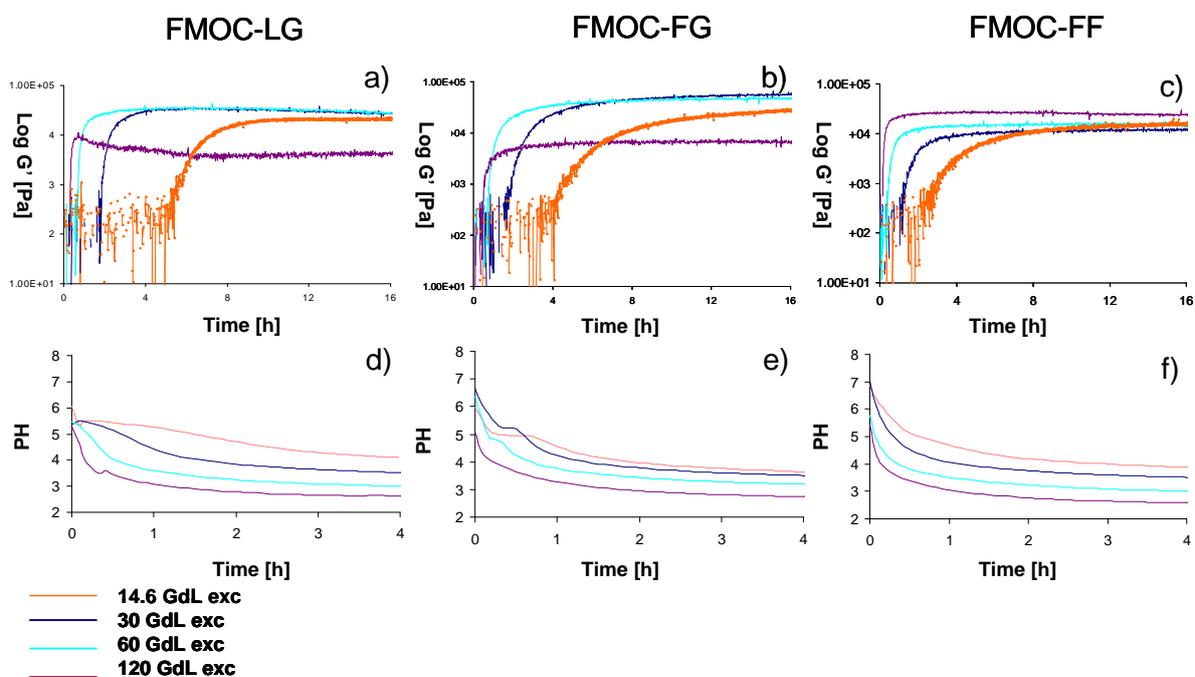


Figure 2. a-c) Variation of the storage modulus (G') upon gelling and d-f) decrease of pH with time for the three Fmoc-dipeptides examined. Data were obtained for solutions with same concentration of peptide and increasing amounts of GdL. Note the different time-scales of the x-axes.

$$\frac{1}{\tau} = k[A]^c \quad (1)$$

$1/\tau$ have been plotted as a function of the GdL concentration (Fig. 3). This estimate of the gelation rate is preferred to that obtainable by the slope of the G' curves in the initial linear region due to the large margins of error for the latter at high gelation rates (especially for the 120 mMol/l GdL excess). It can be shown that for all studied dipeptide solutions the time for gelation τ varies with a power law of the type:

where $[A]$ is the concentration of GdL in solution and k and c are constants estimated empirically and shown for each Fmoc dipeptide in table 1.

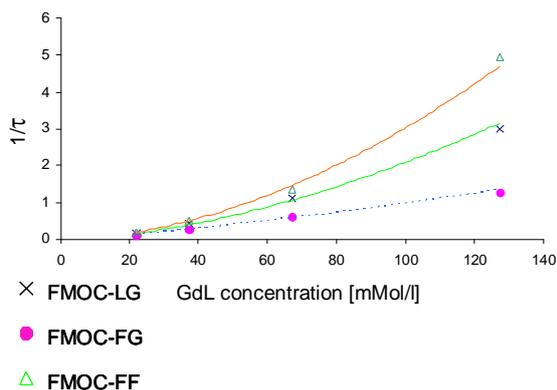


Figure 3. Time shift as a function of GdL excess for the three dipeptides.

Table 1. Fmoc-dipeptides protonation reaction.

| | K | c | R ² |
|---------|---------|--------|----------------|
| FMOC-LG | 7.7E-04 | 1.6951 | 0.996 |
| FMOC-FG | 2.3E-03 | 1.2953 | 0.997 |
| FMOC-FF | 6.2E-04 | 1.8183 | 0.998 |

This is the typical form for the dependence of a chemical reaction on the reactants concentration and a rate constant k . The constant c is the reaction order, which depends on the mechanism of reaction. In our case of gelation triggered by a slowly decreasing pH value, an initial assumption would be that the rate of gelation is proportional to the rate of protonation for the hydrogelators reducing their solubility in water. However the plots in Fig 2 show that gelation occurs significantly after the pH has dropped to its final value. To explain this observation a different reaction mechanism has to be assumed, considering protonated molecules as “building blocks” of the gel network. First the pH drops and the hydrogelators are protonated until this reaction reaches equilibrium. Once this equilibrium is reached, a certain number of molecules will form hydrogen bonds and their electrostatic repulsion will be lower.

Depending on the amount of protonated molecules, rate of self-assembly and gel formation will vary according to a power law. Another explanation is the effect of protonation on the activation energy for the network to form. The protons in solution would lower this energy and the reaction rate would be enhanced.

Differences in modulus of the final gel can be also observed in the curves in Fig. 2 a-c; however, unlike the reaction kinetics, the variations depend in this case on the dipeptide system examined. The modulus of hydrogels containing 120 mMol/l excess is lower for FMOC-LG and FMOC-FG and slightly higher for FMOC-FF compared to that prepared with lower GdL amounts added. Since the vertical shifts signal different gel properties, it can be inferred that not only the kinetics of formation but also the microstructure of the gels changes with increasing GdL concentration. Therefore the gel microstructures have been studied by optical microscopy and cryo-SEM.

Fig. 4 shows the hydrogel networks formed after ≈ 16 hours of gelation upon addition of 14.6 mMol/l GdL excess. The storage modulus curves show that at this stage gelation is complete and the structure formed is stable. At the length-scale of optical microscopy, some fibre bundles are observable for the FMOC-LG (Fig. 4a) while FMOC-FG shows some scattered crystals (Fig. 4b). Depending on the arrangement of the peptide molecules, crystallization may take place³. For all the hydrogels prepared at these conditions the SEM micrographs show that on a smaller scale a fibrillar network is present.

Due to the faster kinetics in the case of a 120 mMol/l GdL excess samples for the structural investigation were already taken after four hours. The SEM micrographs in

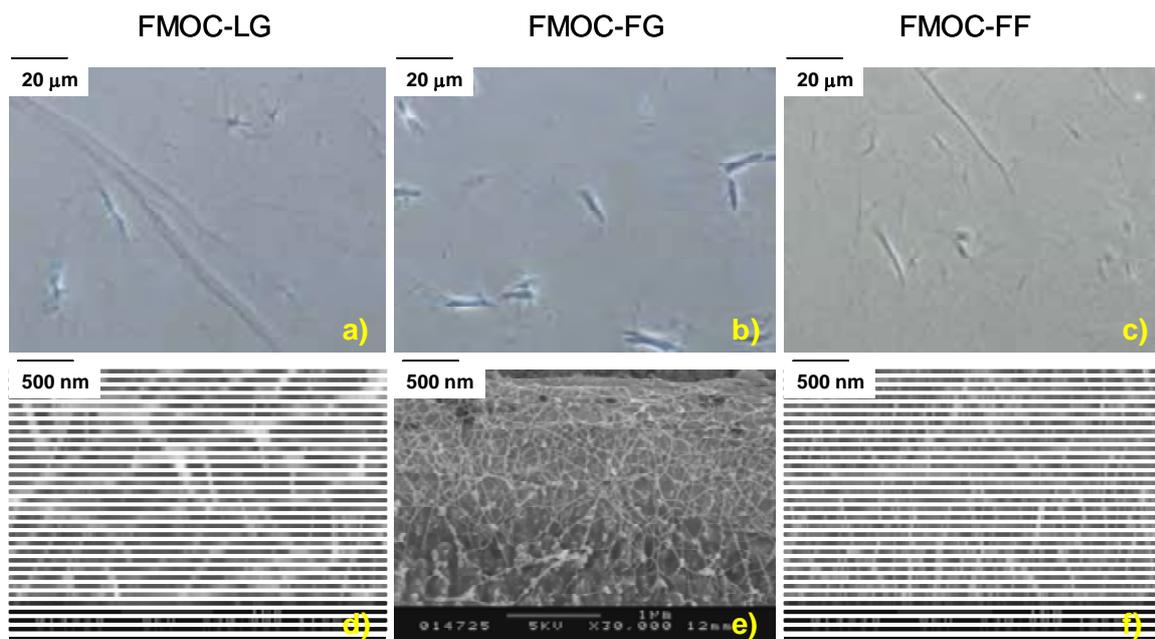


Figure 4. Optical micrographs in phase contrast mode of a) FMOC-LG, b) FMOC-FG, c) FMOC-FF hydrogels after 16 hours of gelation upon 14.6 mMol/l Gdl excess addition. Below d) e) f) the respective SEM micrographs.

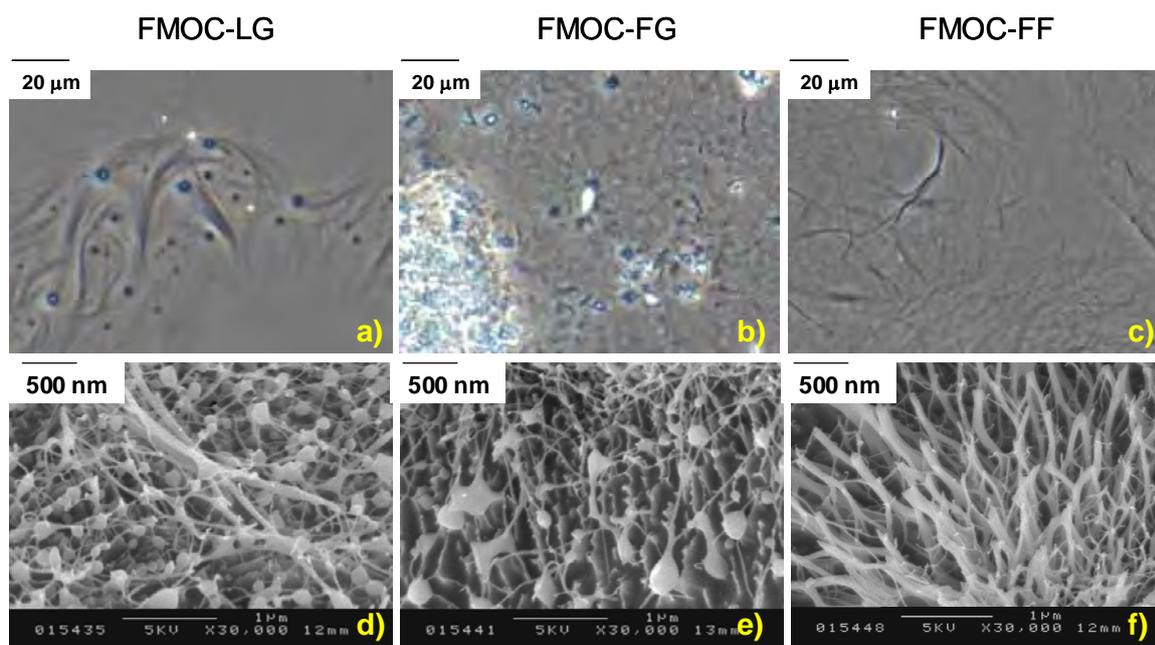


Figure 5. Optical micrographs in phase contrast mode of a) FMOC-LG, b) FMOC-FG, c) FMOC-FF hydrogels after 4 hours of gelation upon 120 mMol/l Gdl excess addition. Below d) e) f) the respective SEM micrographs.

Fig. 4 show that all hydrogels contain a fully formed fibrillar network. However, by comparison of the micrographs in Figs. 4 and 5 the morphology differs from that of the same gelled with lower amounts of GdL for a longer time. FMOC-LG shows some globular structures within the fibres network when the concentration of GdL in solution is increased (Fig. 5d). Differences are more evident for the FMOC-FG hydrogels that together with these structures also contain a larger amount of small crystallites (Fig. 5b). FMOC-FF presents thicker fibers but similar structure at a coarser scale to that obtained by gelation with less GdL excess. These observations suggest that the variation in modulus for the bulk material is caused by structural differences of the hydrogels formed at lower pH, confirming the results in the literature for FMOC-FF^{2, 8}.

Effect of ionic strength on the hydrogel formation

In the following study of the effect of ionic strength, the amount of anions in solution was kept constant during the preparation of the sodium salt by adding a determined molar equivalent of NaOH. The addition of the ionic strength of the solution and the electrostatic forces between molecules and for similar systems salts produced hydrogelation⁶. In order to assess the effect of these forces on gelation as compared to pure hydrogen bonding, part of the GdL excess has been replaced by NaCl to have the same amount of cations but fewer protons. Ionic excess was increased as shown in the diagram in Fig. 6. Gelation has been monitored by oscillatory rheology and the structure has been examined by means of SEM and optical microscopy in phase contrast and polarized mode.

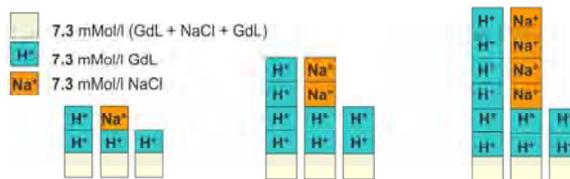


Figure 6. Diagram showing the amounts of GdL and NaCl added to the FMOC dipeptides solution to investigate systematically the effect of increasing ionic strength on gelation.

Results, for sake of clarity, are discussed below only for one of the three model dipeptides. The kinetics of FMOC-LG gel formation with varying ionic strength are shown in Fig. 7 by the curves of G' vs. time. At 14.6 total cations excess (Fig. 7a). At this concentration, kinetics only are affected by the Na⁺ added to the solution: a delay of the gelation occurs but the final hydrogel modulus is comparable to that obtained with the same excess of GdL only. With increasing ionic strength the kinetics of the gel containing NaCl get closer to those of that gelled with an equivalent amount of GdL but a decrease in the modulus occurs (Figs. 7b and 7c). The gel structure has been examined in order to explain this final modulus variation (Fig. 8). After 4 hours of gelation, the sample (45.4 mMol/l) with a high amount of NaCl presents a fully formed fibrillar structure and also some birifringent crystal structures and also some birifringent crystal structures shown in Fig. 8c. Polarized light optical microscopy of the gel containing an equivalent amount of GdL has shown that crystals are not a characteristic of the FMOC-LG hydrogel prepared with GdL only. Such a microstructural change can then explain the differences in modulus observed by oscillatory rheology.

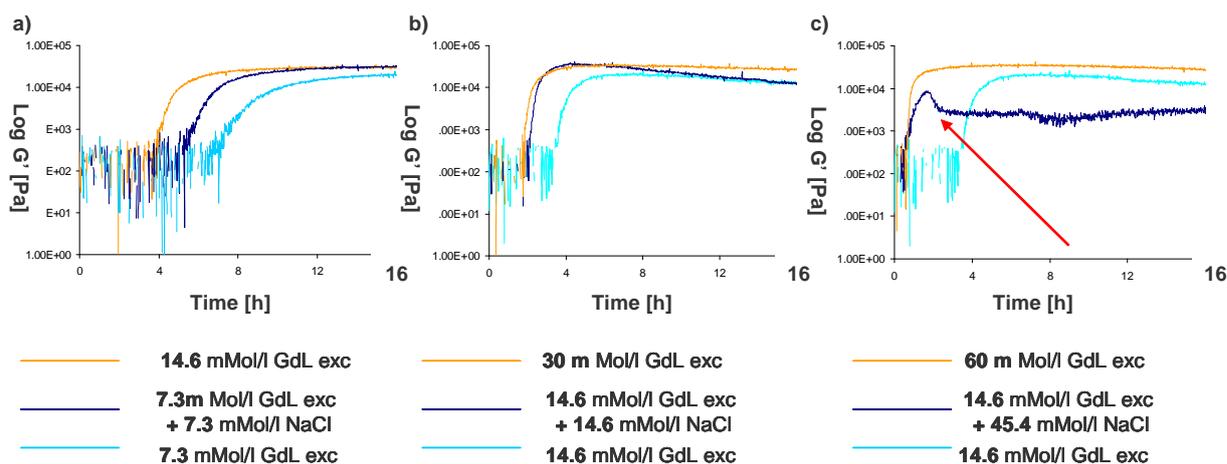


Figure 7. Variation of the storage modulus (G') upon gelling for FMOC-LG with increasing ionic strength. Data were obtained for solutions with same concentration of peptide and various amounts of GdL and NaCl. The total amount of anions in excess in the NaCl containing solution is a) 14.6 b) 30 c) 60 mMol/l. The red arrow indicates the sample examined by microscopy.

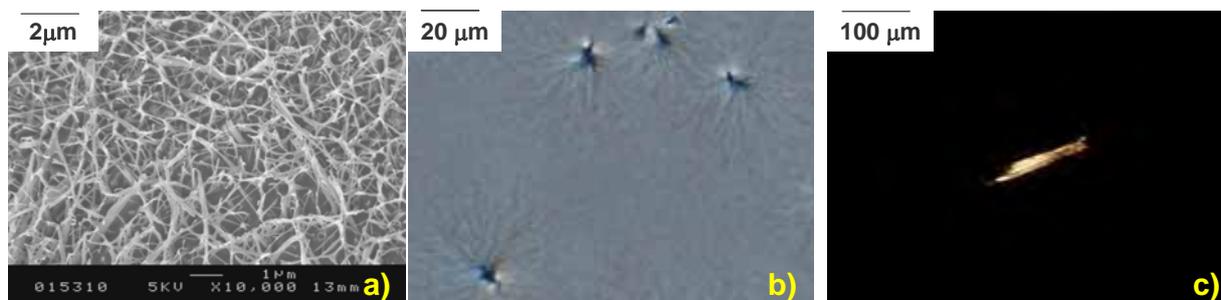


Figure 8. Micrographs showing the structure of a FMOC-LG hydrogel prepared with the addition of 15 mMol/l Gdl and 45 mMol/l NaCl excess, after 4 hours of gelation (see red arrow in Fig. 7). Taken by a) SEM, b) optical microscope in phase contrast mode and c) in polarized light mode showing birefringent crystalline structures.

CONCLUSIONS

For all the dipeptides solutions investigated it was shown that the gelation kinetics are dependent on the amount of GdL excess. A higher GdL excess leads to a lower final pH and causes shifts the equilibrium towards the protonated dipeptides (see Fig. 1) and results in faster gelation. The solution pH affects also the modulus of the final gel, to a different extent depending on the dipeptide structure. Microscopy results showed that such modulus variations can be explained by microstructural changes. Gelation can also be accelerated by increasing the ionic

strength of the dipeptide solution with the addition of salts. If an excess of NaCl is added to the solution replacing some of the GdL (i.e finally replacing protons), the hydrogel formation is generally quicker but it differs from that of solutions containing only GdL and an equivalent amount of cations (see Fig. 7a). As for the GdL, the addition of a large excess of NaCl also has an effect on the final hydrogel structure and appears to favour the formation of crystals in this pH range. The results from this work show that other forces influence the hydrogel formation together with the hydrogen bonds formed with the protonation of the dipeptide terminal carboxylic group.

Tang et al.⁴ reported that protonated and non-protonated molecules assemble into paired fibrils consisting of anti-parallel beta-sheets with ionized terminal carboxylic groups placed on the fibril surface. These ionized carboxylic groups results in negative charges spread on the whole fibril surface and induces repulsion to other fibrils similarly charged. The presence of positive ions in solution can shield this charge allowing self assemble through hydrophobic interaction⁴ or can act as an ion-bridge between molecules and promote their association⁵. The extent of these forces in the self-assembly and the hydrogel structure at given concentrations is also dependent on the molecular structure, so that for each dipeptide a ternary phase diagram [FMOC-XX, H⁺, Na⁺] could be drawn with a sufficient amount of data. However this phase diagram construction is beyond the scope of the present work. This may help explaining the differences in gelling behaviour for the same dipeptide at a given pH between the present work and others in the literature^{3, 5}) as they are possibly related to the method of preparation. Varying the amount of NaOH in the sodium sample by using titration instead of the one-shot method adopted in the present work may affect the dipeptide solution ionic strength. A variation in ionic strength would then determine a different location in the phase diagram and this may produce a different final structure of the hydrogel.

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