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Systematic Exploration of the pH-dependence of a peptide hydrogel

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Dedication to Ron Steer – friend and mentor

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Abstract: Stimuli-responsive peptide gels are a growing class of functional biomaterials that are involved in many applications in research. Here, we present a novel di-peptide hydrogel from the compound Boc-Phe-Trp-OH in various buffer and pH conditions. We examine the effects of different stimuli including temperature and pH on the mechanical strength of the gels through frequency rheology studies. We found that this hydrogelator is highly pH dependent only forming a gel in a narrow range of pH 6-7. This hydrogelator hold promise for the development of new stimuli-responsive biomaterials for specific applications that require this type of specific stimuli.

Keywords: Hydrogel, peptides, stimuli-responsive, pH-dependent gelation, rheology

Draft

Introduction: Peptide-based supramolecular gels are functional materials that have been the subject of research due to their versatility and their wide range of applications in many fields.¹ They can be studied in materials science, catalysis^{2,3}, drug delivery⁴, environmental studies^{5,6}, tissue engineering^{7,8}, and in supramolecular chemistry. The formation of self-assembled gels by low-molecular-weight gelators involves noncovalent interactions such as hydrogen bonding, π - π interactions, hydrophobic interactions, and van der Waal interactions. These noncovalent interactions are responsible for forming self-assembled three-dimensional nanofibrillar network structures. Low molecular weight peptide gelators possess advantages over other polymeric gels due to their ability to form reversible supramolecular assemblies through interactions in response to various stimuli such as light, pH, and temperature.⁹ Amino acids possess a number of interesting features, among them their ability to engage in hydrogen bonding and metal binding makes them attractive targets.¹⁰ Among the 20 common amino acids, tryptophan (Trp) plays a key role in proteins because of its unique properties including having the largest accessible nonpolar surface, an indole NH moiety that is capable of hydrogen-bond donation, and being the amino acid having the greatest electrostatic potential for cation- π interactions.¹¹ As was previously demonstrated, the tryptophan NH can be involved in hydrogen bonding and can play an important role in the supramolecular assembly in the solid state and in the gel state.^{10,12} In addition, the amino acid phenylalanine is heavily reported in the formation of supramolecular gels including hydrogels as they readily self-assemble. Xu et al. reported a metallohydrogelator composed of the amino acids Phe-Phe-Leu.¹³ Not only does this compound self-assemble in water to form a hydrogel but it was also able to exhibit a sol-gel transition upon oxidation of the metal center. In addition, Zhang et al. reported a Fc-Phe-OH compound capable of forming a hydrogel with multi-stimuli responsive properties such as chemical redox and pH.¹⁴ This served

as a guideline when designing novel hydrogels as we incorporated both tryptophan and phenylalanine amino acids into our peptide sequence for the potential of forming hydrogels.

Stimuli responsiveness is an attractive property and is based on a change in peptide secondary structure, solubility and their intermolecular interactions. Temperature is a very common stimulus for gelation and allows for cycling between the gel and sol phases without degradation of the active material. For example, Xu et al. observed that an increase in temperature induced hydrogel formation in Fmoc-Ala-Ala.¹⁵ In this study, temperature as a stimulus for gelation is reported. Another common stimulus includes pH. pH has been reported to be the simplest chemical stimuli that impacts gelation as a slight change in pH can have a large effect on the transition into a gel or back into solution.³ Hydrogen bonding motifs are strongly influenced by pH as the intensity and strength of the hydrogel bonding interactions are affected leading to the collapse of the supramolecular gel when an acid or base is added. For example, Fmoc-Phe-Phe hydrogel formation is triggered by lowering the pH, resulting in the formation of a fibrous network.¹⁶

Here, we report a dipeptide gelator and its ability to form a hydrogel in response to various pH as a stimulus. The rheological properties including self-healing abilities were explored and these mechanical properties were compared in different pH values to show the effect. The intermolecular hydrogen bonding interactions were investigated using circular dichroism (CD) by comparing the peaks in gel and sol state. In addition, the morphology of the hydrogelator was explored using transmission electron microscopy (TEM) and was reported to have an optimal nanofiber matrix, which is commonly reported for peptide gels. These gels were designed with future experiments in mind including the exploration of the gels as a biomimetic scaffold for the entrapment of biological compounds. These gelator molecules hold promise for

the development of new self-assembling biomaterials, and for the potential use in a variety of biomedical applications.

Results and Discussion:

Stimuli-responsive gelation studies

Boc-Phe-Trp-OH (**1**), was synthesized using standard carbodiimide coupling procedures using the amino acids phenylalanine and tryptophan, in the presence of HOBt.¹⁷ A synthetic scheme of the peptide is shown in figure 1 and its full characterization is found in the SI.

Figure 1: Synthetic scheme of compound **1**, Boc-Phe-Trp-OH.

The self-assembling behavior of the di-peptide conjugate was explored in a variety of buffers. When a hot solution of compound **1** in buffer cooled to room temperature, a viscoelastic gel was obtained. Compound **1** was found to form a gel in various buffer conditions in a specific pH range between 6-7 (figure 2). Acidic pH or basic pH did not support hydrogel formation. The gelation information for compound **1** including the minimum gelation concentration (MGC) and the melting temperature of the gel (T_{gel}) are reported in Table 1. A list of the possible buffers tried and a range of pH can be found in Table S1.

Figure 2: Image showing solutions of Boc-Phe-Trp-OH at from pH 3-9, from left to right. Hydrogel formation was only possible in a narrow pH range of 6-7. Inverted vials at pH 6 and 7 show stable hydrogel formation. All buffers were tested with the compound at each pH, the vials shown here are in Phosphate buffer (0.1 M K_2HPO_4) except for the vial at pH 7 (HEPES buffer).

Table 1: MGC, T_{gel} , and $\tan \delta$ values of compound **1** hydrogels in various buffer and pH conditions.

Compound 1 Hydrogel Buffer	pH	MGC	T_{gel}	$\tan \delta$
0.1 M K_2HPO_4	6	1.1 %	50 °C	0.064
0.01 M K_2HPO_4	6	1.1 %	57 °C	0.065
0.1 M HEPES	7	2.3 %	65 °C	0.089
0.1 M TRIS	7	1.6 %	54 °C	0.072

Rheology Studies

Frequency sweep experiments were performed to study the mechanical strength of the gels (Figure 3). For a typical viscoelastic gel, the storage modulus G' remains higher than the loss modulus G'' .¹⁸ From the data shown in Figure 3 a-d, the frequency sweep experiment for the compound was shown to be a gel as G' was greater than G'' . The $\tan \delta$ value (G''/G') is a measure of gel elasticity that measures the energy loss owing to viscous flow compared to

energy stored.¹⁹ A lower $\tan \delta$ value indicates a stronger gel. Here, we compared the mechanical properties of the Phe-Trp gel at different pH to see the effects of the stimulus. It was found that the hydrogel in phosphate buffer at pH 6 was the strongest indicated by the lowest $\tan \delta$ value of 0.064 for 0.1 M and a $\tan \delta$ value of 0.065 for 0.01 M (table 1). The gel no longer formed when the pH decreased or increased proving that it is highly pH dependent and sensitive. In HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) and TRIS (tris(hydroxymethyl)aminomethane) buffer, however, the gels only formed at pH 7 further proving is pH sensitivity. These hydrogels gave a $\tan \delta$ value of 0.089 and 0.072 for HEPES and TRIs buffer, respectively (table 1)

Figure 3: Frequency experiments of compound **1** in a) 0.1 M phosphate buffer pH 6, b) 0.01 M phosphate buffer pH 6, c) 0.1 M TRIS buffer pH 7, d) 0.1 M HEPES buffer pH 7. Step-strain experiment of compound **1** e) in 0.1 M phosphate buffer pH 6 exhibiting self-healing properties. f) in HEPES buffer pH 7 exhibiting self-healing properties.

Self-healing experiments were also performed, in which strain was applied to the gel causing the gel to revert to a solution phase. Self-healing gels that can restore their functionalities and structures after damage have been developed as “smart” soft materials.²⁰ The strain was then removed to see if the gel reformed. Interestingly compound **1** exhibited self-healing abilities and the data in Figure 3 e-f confirms that compounds **1** forms a self-healing gel. In the first cycle in

which 0.1% strain is applied G' is greater than G'' , confirming it is a gel. In the second cycle, 100% strain is applied and we observed that G'' was then greater than G' , causing it to go to sol state. When the strain was removed and set to 0.1% in the third cycle, the storage modulus was again greater. This behavior is reversible and repeated stress cycles do not alter the gel's ability to reform into a gel.

Supramolecular state

To further confirm the self-assembled state of the compound, circular dichroism (CD) spectroscopy was performed. CD spectroscopy is a valuable technique that can be used to determine molecular chiral arrangements in a self-assembled state.²¹ CD experiments in the gel and sol state of each compound were performed to study the effect of molecular structure on supramolecular aggregation (Figure 4).²² The intermolecular interactions give rise to CD bands that are much more intense in the self-assembled state. Bands below 300 nm arise from chiral amino acids. Individual molecules assemble into larger chiral supramolecular aggregates which give rise to higher ellipticity.¹⁰ As shown in figure 4, the CD spectra for the gel state (blue) shows a much more intense spectrum peaks compared to sol state (black). The increase in intensity is due to the formation of a supramolecular structure.

Figure 4: CD spectrum of compound **1** in sol and gel state. An increase in intensity of the peaks in supramolecular state is seen compared to the sol state.

Morphological Investigation

To investigate the morphological features of the self-assembling peptide conjugates, transmission electron microscopy (TEM) studies were performed (Figure 5). For this experiment, compound **1** was dissolved in phosphate buffer, followed by a temperature-triggered gelation. TEM images of compound **1** show the presence of a nanofiber matrix which is optimal for hydrogel formation. Fibril structures have also been reported as the main morphological structure of relatively strong gels.^{8,23–27}

Figure 5: Inverted vial test of the compound **1** as a hydrogel and a TEM image of the gel showing a nanofiber matrix at room temperature.

Conclusion

The construction of three self-healing supramolecular gels from the amino acids phenylalanine and tryptophan was investigated in this study. The compound exhibited many interesting properties including supramolecular gel formation, self-healing abilities, and temperature and pH response. A nanofiber network formation is reported as the main morphology, which is optimal for peptide gels. We also further emphasize the attractive properties of the amino acid Trp as it is an interesting amino acid that is not heavily reported in gels. The highly pH sensitive nature of

the hydrogel demonstrates a very strong hydrogel that can be used in very specific conditions for certain applications that require this pH range. This gelator molecule holds promise for the development of new self-assembling biomaterials

Experimental

General

Boc and methyl esters of amino acids were purchased from Advanced ChemTech, Louisville, KY, USA. Chemicals were used as received without further purification.

Compound **1** dipeptide was synthesized using the standard 1-ethyl-3-(3'-dimethylaminopropyl)carbodiimide (EDC)/1-hydroxybenzotriazole (HOBt) method. Boc-Phe-OH (8 mmol) was dissolved in DCM and HOBt (4 mmol, 0.678 g) and EDC (4 mmol, 0.767 g) were added to the reaction mixture at 0 °C, and the reaction mixture was stirred for 15 min in ice. 5 mmol of Trp-OME was added to the solution and was stirred for 24 hours at r.t. After a standard aqueous workup consisting of sequential washing with saturated aqueous NaHCO₃, aqueous 10% citric acid solution, and brine, followed by collection and drying of the organic layer, the crude material was purified by column chromatography on silica gel to afford compound **1** as a white solid (2.234 g, 84% yield). ¹H NMR (500 MHz, [D₆] dimethyl sulfoxide): 12.70 (s, 1H, OH) 10.88 (s, 1H; indole NH), 8.12 (s, 1H; amide NH), 7.54 (d, J=7.6 Hz, 1H; amide NH), 7.35 (d, J=37.4 Hz, 1H; H-Trp), 7.22 (m, 5H; H-Phe), 7.18 (s, 1H; indole-NH), 7.07 (t, J=7.0 Hz, 1H; H-Trp) 6.99 (t, J=7.0 Hz, 1H; H-Trp), 6.89 (d, J=7.0 Hz, 1H; amide NH), 4.52 (m, 1H; CH α), 4.19 (m, 1H; CH α), 3.19 (m, J=8.1 Hz, 1H; CH₂ β), 3.10 (m, J=8.1 Hz, 1H; CH₂ β), 2.93 (m, J=8.1 Hz, 2H; CH₂ β), 2.68 (m, J=8.1 Hz, 2H; CH₂ β), 1.28 (s, 9H; (CH₃)₃); ¹³C NMR (125 MHz, [D₆] dimethyl sulfoxide): δ =173.64 (1C; C=O), 172.11 (1C; C=O), 155.59 (1C; C=O), 138.58 (1C; benzene), 136.51 (1C; indole), 129.64 (3C; benzene), 128.42 (3C; 2C-

indole, 1C-benzene), 127.71 (1C; indole), 118.82 (2C; indole), 111.79 (1C; indole), 109.98 (1C; indole), 78.50 (1C; C-CH₃), 56.16 (1C; α -CH), 53.31 (1C; α -CH), 28.66 (1C; β -CH₂), 28.51 (1C; β -CH₂), 27.58 ppm (3C; (CH₃)₃); FTIR: $\tilde{\nu}$ = 3284 (amide N-H), 1718 (ester C=O), 1658 cm⁻¹ (amide C=O); ESI-MS: m/z: 451.2107 [M]; Found m/z 474.1999 ([C₂₅H₂₉N₃O₅]+Na)⁺; elemental analysis for C₂₅H₂₉N₃O₅: C 66.50, H 6.47; N 9.31, O: 17.72; found: C 66.48, H 6.50, N 9.34.

Nuclear Magnetic Resonance Spectroscopy

Compounds **1** was characterized using NMR spectroscopy on a Bruker 500 MHz spectrometer. The sample was dissolved in [D₆] dimethyl-sulfoxide at a concentration of 2 mM. Spectra are shown in the Supporting Information.

FTIR Spectroscopy

FTIR spectra of the compound were recorded in the range 3400– 1400 cm⁻¹ using a Bruker Alpha FTIR spectrometer equipped with a diamond for attenuated total reflection. A wet viscous sample was prepared by dissolving compound **1** in phosphate buffer, forming the gel, then keeping the samples under vacuum to dry.

Transmission Electron Microscopy

The morphology of the self-assembled compound **1** was studied using TEM. The sample was prepared by depositing a small amount of a viscous solution of compound **1** in phosphate buffer, followed by sonication, on a copper TEM grid (300 mesh size) coated with Formvar and a carbon film. The grid was allowed to dry by slow evaporation in air and then further dried in a vacuum for 24 hours. Images were acquired using a Hitachi 7500 transmission electron microscope at room temperature.

Circular Dichroism Spectroscopy

CD spectroscopy was used for determining the chiral molecular arrangement of its supramolecular assemblies, in gelling (phosphate buffer) and non-gelling (methanol) solvents. CD spectra were recorded between 165 and 600 nm using a Jasco J-810 spectrometer. Experiments were performed by placing the gel-like viscous materials obtained from compound **1** into a quartz cuvette of 1 mm path length.

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References

- (1) Falcone, N.; Kraatz, H. B. *Chem. A Eur. J.* **2018**, *24*, 14316–14328.
- (2) Wang, Q.; Yang, Z.; Gao, Y.; Ge, W.; Wang, L.; Xu, B. *Soft Matter* **2008**, *4*, 550–553.
- (3) Du, X.; Zhou, J.; Shi, J.; Xu, B. *Chem. Rev.* **2015**, *115* (24), 13165–13307.
- (4) Altunbas, A.; Lee, S. J.; Rajasekaran, S. A.; Schneider, J. P.; Pochan, D. J. *Biomaterials* **2011**, *32*, 5906–5914.
- (5) Basak, S.; Nanda, J.; Banerjee, A. *J. Mater. Chem.* **2012**, *22* (23), 11658.
- (6) Adhikari, B.; Palui, G.; Banerjee, A.; et al. *Soft Matter* **2009**, *5*, 3452–3460.
- (7) Jayawarna, V.; Richardson, S. M.; Hirst, A. R.; Hodson, N. W.; Saiani, A.; Gough, J. E.; Ulijn, R. V. *Acta Biomater.* **2009**, *5*, 934–943.
- (8) Rodriguez, A. L.; Wang, T. Y.; Bruggeman, K. F.; Horgan, C. C.; Li, R.; Williams, R. J.; Parish, C. L.; Nisbet, D. R. *J. Mater. Chem. B* **2014**, *2*, 7771–7778.
- (9) Panda, J. J.; Mishra, A.; Basu, A.; Chauhan, V. S. *Biomacromolecules* **2008**, *9*, 2244–2250.
- (10) Adhikari, B.; Afrasiabi, R.; Kraatz, H. *Organometallics* **2013**, *32*, 5899–5905.

- (11) Yitamben, E. N.; Clayborne, A.; Darling, S. B.; Guisinger, N. P. *Nanotechnology* **2015**, *26*, 1–6.
- (12) Falcone, A. N.; Basak, S.; Dong, B.; Syed, J.; Ferranco, A.; Lough, A.; She, Z.; Falcone, N.; Basak, S.; Dong, B.; Syed, J.; Ferranco, A. *Chempluschem* **2017**, *82*, 1282–1289.
- (13) Zhang, Y.; Zhang, B.; Kuang, Y.; Gao, Y.; Shi, J.; Zhang, X. X.; Xu, B. *J. Am. Chem. Soc.* **2013**, *135*, 5008–5011.
- (14) Sun, Z.; Li, Z.; He, Y.; Shen, R.; Deng, L.; Yang, M.; Liang, Y.; Zhang, Y. *J. Am. Chem. Soc.* **2013**, *135*, 13379–13386.
- (15) Zhang, Y.; Gu, H.; Yang, Z.; Xu, B. *J. Am. Chem. Soc.* **2003**, *125*, 13680–13681.
- (16) Tang, C.; Smith, A. M.; Collins, R. F.; Ulijn, R. V.; Saiani, A. *Langmuir* **2009**, *25*, 9447–9453.
- (17) Benoiton, N. L.; Kuroda, K.; Chen, F. M. F. *Int. J. Pept. Protein Res.* **1980**, *15*, 475–479.
- (18) Orbach, R.; Mironi-Harpaz, I.; Adler-Abramovich, L.; Mossou, E.; Mitchell, E. P.; Forsyth, V. T.; Gazit, E.; Seliktar, D. *Langmuir* **2012**, *28*, 2015–2022.
- (19) Yan, C.; Pochan, D. J. *Chem. Soc. Rev.* **2010**, *39*, 3528–3540.
- (20) Wei, Z.; Yang, J. H.; Zhou, J.; Xu, F.; Zrínyi, M.; Dussault, P. H.; Osada, Y.; Chen, Y. M. *Chem. Soc. Rev.* **2014**, *43*, 8114–8131.
- (21) Kirin, S. I.; Kraatz, H.-B.; Metzler-Nolte, N. *Chem. Soc. Rev.* **2006**, *35* (4), 348–354.
- (22) Zhou, M.; Smith, A. M.; Das, A. K.; Hodson, N. W.; Collins, R. F.; Ulijn, R. V.; Gough, J. E. *Biomaterials* **2009**, *30*, 2523–2530.
- (23) Adhikari, B.; Kraatz, H.-B. *Chem. Commun.* **2014**, *50*, 5551–5553.
- (24) Adhikari, B.; Lough, A. J.; Barker, B.; Shah, A.; Xiang, C.; Kraatz, H.-B. *Organometallics* **2014**, *33*, 4873–4887.

- (25) Ramos Sasselli, I.; Halling, P. J.; Ulijn, R. V.; Tuttle, T. *ACS Nano* **2016**, *10* (2), 2661–2668.
- (26) Yu, Y.; Wang, S.; Jia, L.; Zhou, M.; Pan, Q.; Zhai, Y.; Wang, C. *J. Sol-Gel Sci. Technol.* **2016**, *78* (1), 218–227.
- (27) Edwards, W.; Smith, D. K. *J. Am. Chem. Soc.* **2014**, *136* (3), 1116–1124.

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