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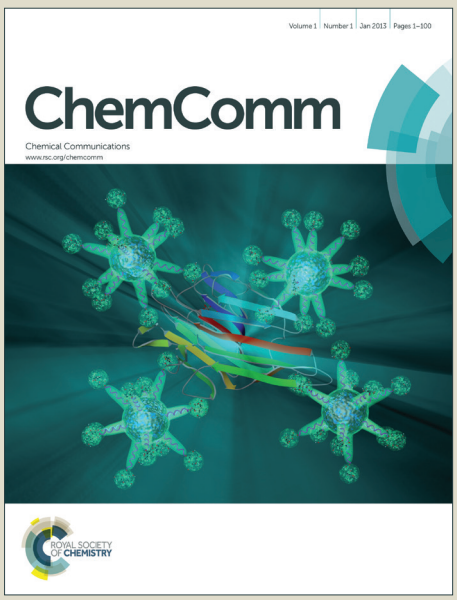

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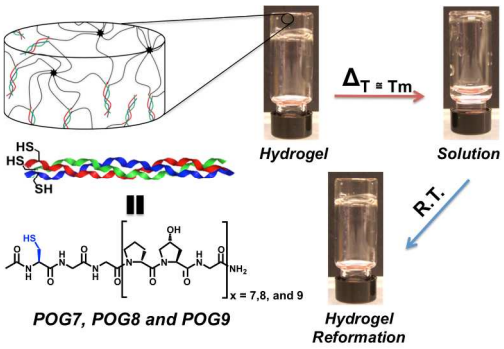

Collagen peptide, PEG-based hydrogels with tuneable thermosensitive properties are validated as stimuli-responsive materials.

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Tuning the Thermosensitive Properties of Hybrid Collagen Peptide-Polymer Hydrogels

Cite this: DOI: 10.1039/x0xx00000x

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Received 00th January 2012,
Accepted 00th January 2012

DOI: 10.1039/x0xx00000x

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A hybrid hydrogel based on collagen-mimetic peptides has been designed with tunable thermosensitive properties. By changing the number of POG repeats within the collagen peptide sequence, the thermal stability of the triple helical physical crosslinks of a peptide-polymer conjugate can be altered, thus tuning the stiffness of the hydrogel as a function of temperature. This report focuses on three different thermally responsive collagen peptide, PEG-based hydrogels and validates their use as stimuli-responsive materials.

Hydrogels have become one of the most translatable materials for medical applications in the field of tissue engineering.[1] Their easy formation and malleability are two features that have been exploited for exogenous cell growth and for use as implants to promote the repair and regeneration of damaged tissue.[2] Many early hydrogels used synthetic polymers exclusively, including poly(ethylene)glycol (PEG), poly(2-hydroxyethylmethacrylate) (pHEMA) or poly(N-isopropylacrylamide (pNIPAAM). These polymers can be chemically tuned to obtain desired mechanical properties for particular applications,[2c] but lack the endogenous cell signalling cues that biomolecules provide. Hydrogels based on natural proteins such as fibrin, collagen and elastin, and carbohydrates such as hyaluronic acid and chitosan have been employed to mimic a typical bioenvironment,[3] but these biopolymers are too chemically complex for site-selective modification and are usually mechanically weak.

An emerging strategy in the field is the inclusion of biomimetic peptide sequences, to replace the use of proteins from natural sources, into synthetic polymers to promote hydrogel formation through non-covalent interactions.[4] This has yielded peptide-polymer hybrid materials that incorporate various mimetic peptide structures, such as coiled-coil,[5] elastin β-sheet[6] and collagen triple helix[7] sequences. Inspired by its role as a major component of the extracellular matrix,[8] collagen mimetic peptides alone have also been used for the creation of an assortment of biomaterials, including hydrogels.[9] Herein, we present a strategy whereby the thermal stability of collagen mimetic peptides is used to tune the physical properties of a collagen peptide-polymer hydrogel system.

The design of hydrogels with controllable thermoresponsive behavior involved the integration of a star-PEG polymer with collagen peptide triple helices of varying thermal stability (Figure 1). Through judicious use of conjugation chemistry, the collagen peptides would act as the physical crosslinks for the polymer system through triple helix formation. Specifically an

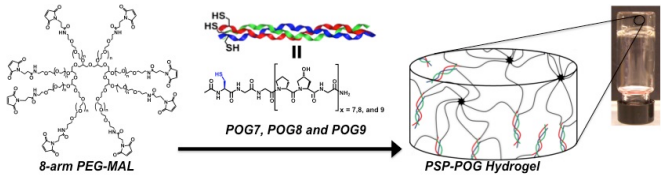

Figure 1 Design of thermosensitive PSP-POG hydrogels based on two components: an 8-arm PEG-MAL polymer and Cys-modified collagen triple helical peptides with varying POG repeats.

8-arm PEG multi-arm polymer, functionalized at each arm with a maleimide moiety (PEG-MAL), would be treated with Cys-modified collagen peptides with varying Pro-Hyp-Gly (POG) repeats to produce a collagen peptide-PEG star polymer conjugate (PSP-POG) (Figure 1). In a preliminary study, one such collagen peptide (**POG8**) promoted gelation within the polymer conjugate through the physical crosslinks of the collagen triple helix.[10] This hydrogel displayed reversible formation of a liquid-like state near the melting temperature (T_m) of the collagen triple helix. The porous hydrogel network was also found to sustain cell viability using human mesenchymal stem cells.[10] In this work, we have expanded our exploration of the thermosensitive mechanical properties of hydrogels by investigating collagen peptide sequences that each posses a unique triple helical melting temperature, so as to assess the ability to control the thermal stability of the resulting hydrogels.

The collagen peptides were designed to contain varying units of POG repeats, since POG is the most common amino acid triad found in natural collagen.[11] This yielded **POG7**, **POG8** and **POG9**, where the number corresponds to the quantity of POG triplets in the peptide sequence (Figure 1). The N-terminus of each peptide contained a Cys-Gly-Gly triad for subsequent attachment to the 40kDa 8-arm PEG-MAL polymer via Michael addition. The peptides were synthesized using a solid phase strategy on a Rink amide resin. After cleavage from the solid support and concomitant removal of the protecting groups, the peptides were purified to homogeneity by reverse phase HPLC and analysed by MALDI mass spectrometry (See Table S1). Circular dichroism (CD) experiments confirmed that each Cys-modified peptide (200 μM in PBS buffer) adopted a stable triple helical conformation, with melting temperatures (T_m) that increased with increasing peptide length (Table 1 and Figure S3). These values are consistent with similar length collagen peptides published in the literature.[9d, 12]

Table 1. Summary of the melting temperatures (T_m) of the collagen peptides free in solution and within the PSP-POG conjugates as determined by CD.

Triple Helix Melting Temperature (T_m)		
	Free Peptide	PSP-POG
POG7	41 °C	44 °C
POG8	53 °C	56 °C
POG9	61 °C	63 °C

The PSP-POG conjugates were prepared by treating PEG-MAL (5% w/v) with each peptide (5 mM) in PBS buffer to yield 4% **PSP-POG7**, **PSP-POG8** and **PSP-POG9** conjugates. Each conjugate formed an immediate hydrogel at room temperature (Figure 1 and see Supporting Information for a detailed hydrogel preparation protocol). By diluting each hydrogel (1:25 dilution in PBS buffer) the T_m values for the triple helices within the PSP-POG conjugates were determined (Table 1 and see Supporting Information for a detailed hydrogel CD solution preparation). As observed for the free collagen peptides, increasing the number of POG units led to an increase in the T_m values, as the longer triple helices in the conjugates also became more resistant to denaturation. The PSP-POG conjugates displayed a slightly higher T_m (2-3°C) as compared to their free peptide counterparts (Table 1 and Figure S3). Overall, the T_m values for the three polymer-peptide conjugates were ~10 degrees apart, a difference that may impart unique thermosensitive properties to the hydrogels.

The conjugates were evaluated using rheology at constant temperature to determine the storage modulus (G') or stiffness for each of the PSP-POG hydrogels. Since all three peptides formed stable triple helices at room temperature within the hydrogels, the storage modulus was expected to be similar among the three gels. By conducting a frequency sweep experiment, it was confirmed that the three hydrogels possessed G' values between 640-700 Pa (Figure 2A). By applying an increasing amount of oscillation stress, the hydrogels lost all solid-like properties at an oscillation stress value of 500 Pa, where the G" values (loss modulus) became greater than the G' values (Figure 2B). These losses in the storage modulus (stiffness) are likely due to the denaturation of the collagen triple helices at the high-applied pressures. Once the pressure was released, each hydrogel returned to its initial modulus. Overall, the three 4% PSP-POG hydrogels possessed a similar stiffness after formation, demonstrating that the length of the collagen peptide does not affect the stiffness of the gels, as long as stable triple helices form at room temperature.

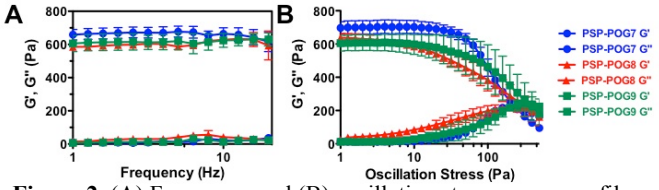

Figure 2. (A) Frequency and (B) oscillation stress sweep profiles comparing the 4% PSP-POG hydrogels using rheometry at room temperature.

Since the stability of each of the collagen peptide triple helices was unique for the three hydrogels, we wished to investigate the stiffness of each hydrogel as a function of temperature by performing rheological temperature sweep cycles. In this experiment, we formed the hydrogel at room temperature and slowly increased the temperature of the sample on the rheometer plate while simultaneously measuring the change in G'. As the triple helix is denatured with increasing temperature, we would expect the gel to weaken into a liquid-like state, but at unique temperatures for each hydrogel depending on the length of the collagen peptide attached to the polymer. Upon cooling, the triple helix should re-anneal and restore the hydrogel back to its initial stiffness (Figure 3A). Upon heating the 4% **PSP-POG7** hydrogel (denaturation cycle), we observed that the storage modulus remained fairly constant up to 35°C (Figure 3B). Near the melting temperature of the peptide triple helix within the hydrogel (44°C) there was

a significant loss in the storage modulus , and at 50°C the hydrogel had completely melted into a liquid-like state. In contrast, the storage modulus of the 4% **PSP-POG9** hydrogel remained fairly constant up to 50°C, and the liquid-like state was only achieved at 70°C, approximately 7 degrees above the T_m of **PSP-POG9** triple helix. The melting behaviour of the **PSP-POG8** hydrogel was intermediate between the **POG7** and **POG9** gels, with a liquid-like state obtained at 60°C.

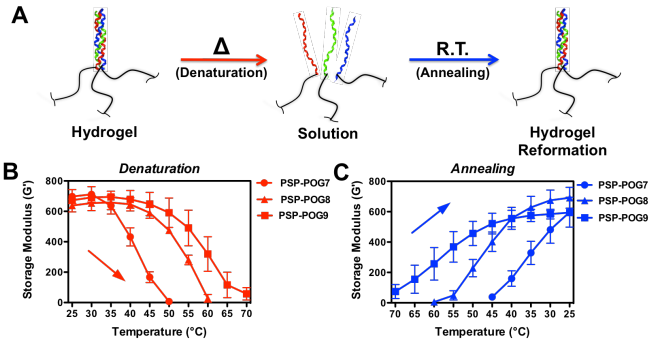

Figure 3 (A) Schematic of the denaturation-annealing cycle of the triple helix within the hydrogel. Effect of temperature on the storage modulus of the 4% PSP-POG hydrogels (B) with increased temperature and (C) with cooling back to room temperature.

Since the T_m value indicates the temperature at which half of the triple helices are unfolded, these data demonstrate that more than 50% of the physical crosslinks of the PEG network must be maintained as stable triple helices for the hydrogels to remain in a solid-like state (wherever G' > G"). After the hydrogels are gradually cooled back to room temperature, the unfolded collagen peptide population can re-anneal into stable triple helices as indicated by the rise in storage modulus during the annealing cycle (Figure 3C). By combining both of the denaturation and annealing curves from Figures 3B and 3C, we observed that the stiffness values at a particular temperature during the annealing cycle do not match those obtained during the denaturation cycle, giving rise to characteristic hysteresis loops for each hydrogel (Figure S5). This is likely due to the rapid drop in temperature (5°C/min) during the annealing cycle, thus not allowing sufficient time for proper triple helical folding,[13] until the hydrogel cools completely to room temperature. At this point, the G' values are near their initial values prior to denaturation (Table 2). Overall, This data conclusively supports the hypothesis that the T_m of the collagen triple helix directly affects the thermal stability of the hydrogel.

Table 2 Summary of the storage moduli (G') of the three 4% PSP-POG hydrogels before and after thermal annealing.

Hydrogel	G' (before Δ) [Pa]	G' (after cooling) [Pa]
4% PSP-POG7	697 ± 46	597 ± 98
4% PSP-POG8	673 ± 42	692 ± 68
4% PSP-POG9	638 ± 4	595 ± 20

We also investigated the internal morphology of the PSP-POG hydrogels by cryogenic scanning electron microscopy (Cryo-SEM). The 4% **PSP-POG7** and **PSP-POG9** materials were found to consist of a highly cross-linked network of pores (Figure 4), as has previously observed with the **PSP-POG8** hydrogel.[10] The size of the pores was poly-disperse with an average diameter of 4 to 10 µm, with a lesser quantity of smaller (2 µm) and larger (25 µm) pores. Higher magnification micrographs showed small cavities inside of the pores, potentially showing incomplete crosslinking of collagen peptides or shear damage during the cryogenic process (Figure S5). Overall, the dimensions of the pores make the scaffolds adequate for 3D encapsulation and growth of cells *in vitro*.[2c]

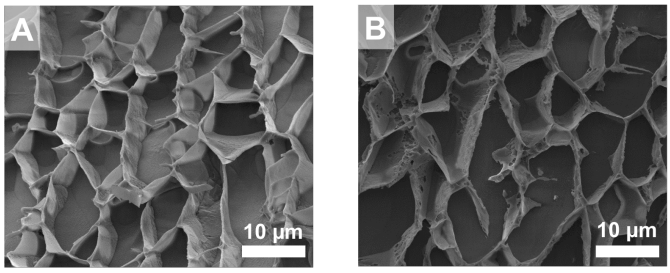

Figure 4 (A) Cryo-SEM micrographs of the porous network formed within the 4% **PSP-POG7** and (B) 4% **PSP-POG9** hydrogels.

In summary, we have demonstrated that versatile, thermosensitive hydrogels can be obtained simply by tuning the collagen peptide sequence. By conjugating the collagen-based peptides **POG7-9** (24, 27 and 30 amino acids in length) to the multi-arm PEG polymer, the initial mechanical properties of the resulting hydrogels were maintained, demonstrating the importance of a stable triple helix as the physical crosslink for the hydrogel. As the length of the peptide was increased, however, the thermal stability of the materials was altered significantly, with the longer, more stable triple helical peptides giving rise to a more thermally stable hydrogel. This thermoresponsive behaviour has potential benefit for the delivery of cargo, such as proteins or cells, encapsulated within the hydrogels in *in vivo* systems for tissue repair and regeneration.[14] In this way, encapsulated cargo within the PSP-POG hydrogels could be released in a time- and temperature-dependent manner through collagen peptide design. Chemical modifications to the hydrogels could also be easily accomplished, such as the addition of cell adhesion sequences,[15,16] without compromising the storage modulus of the hydrogel as long as stable, collagen peptide triple helices are formed.

We are grateful to the NSF (1213948-CHE) for support of this research. We acknowledge D. Sherman for assistance with Cryo-SEM and A. Panitch for providing samples of PEG-MAL.

Notes and references

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Electronic Supplementary Information (ESI) available: See DOI: 10.1039/c000000x/

1 (a) D. G. Wallace and J. Rosenblatt, *Adv. Drug Deliver Rev.*, 2003, **55**, 1631; (b) B. V. Slaughter, S. S. Khurshid, O. Z. Fisher, A. Khademhosseini and N. A. Peppas, *Adv. Mater.*, 2009, **21**, 3307.
2 (a) J. Thiele, Y. Ma, S. M. Bruekers, S. Ma and W. T. Huck, *Adv. Mater.*, 2013, **26**, 125 ; (b) N. Annabi, A. Tamayol, J. A. Uquillas, M. Akbari, L. E. Bertassoni, C. Cha, G. Camci-Unal, M. R. Dokmeci, N. A. Peppas and A. Khademhosseini, *Adv. mater.* , 2014, **26**, 85; (c) M. W. Tibbitt and K. S. Anseth, *Biotechnol. Bioeng.*, 2009, **103**, 655; (d) P. de Vos, H. A. Lazarjani, D. Poncelet and M. M. Faas, *Adv. Drug Deliver Rev*.
3 N. C. Hunt and L. M. Grover, *Biotechnol. Lett.*, 2010, **32**, 733.
4 (a) A. M. Jonker, D. W. P. M. Löwik and J. C. M. van Hest, *Chem. Mater.*, 2011, **24**, 759; (b) J. Kopecek and J. Yang, *Angew. Chemie*, 2012, **51**, 7396; (c) P. M. Kharkar, K. L. Kiick and A. M. Kloxin, *Chem. Soc. Rev.*, 2013, **42**, 7335; (d) G. W. M. Vandermeulen and H. A. Klok, *Macromol. Biosci.*, 2004, **4**, 383; (e) J. Y. Shu, B. Panganiban and T. Xu, *Annu. Rev. Phys. Chem.*, 2013, **64**, 631; (f) J. Kopecek and J. Yang, *Acta biomater.*, 2009, **5**, 805.
5 (a) W. A. Petka, J. L. Harden, K. P. McGrath, D. Wirtz and D. A. Tirrell, *Science*, 1998, **281**, 389; (b) J. Yang, C. Xu, C. Wang and J. Kopecek, *Biomacromol.*, 2006, **7**, 1187; (c) B. D. Olsen, J. A. Kornfield and D. A. Tirrell, *Macromol.*, 2010, **43**, 9094.
6 (a) I. W. Hamley, I. A. Ansari, V. Castelletto, H. Nuhn, A. Rosler and H. A. Klok, *Biomacromol.*, 2005, **6**, 1310; (b) L. C. Wu, J. Yang and J. Kopeček, *Biomater.*, 2011, **32**, 5341; (c) T. S. Burkoth, T. L. S. Benzinger, D. N. M. Jones, K. Hallenga, S. C. Meredith and D. G. Lynn, *J. Amer. Chem. Soc.*, 1998, **120**, 7655.
7 (a) P. J. Stahl, N. H. Romano, D. Wirtz and S. M. Yu, *Biomacromol.*, 2010, **11**, 2336; (b) M. Matsusaki, R. Amekawa, M. Matsumoto, Y. Tanaka, A. Kubota, K. Nishida and M. Akashi, *Adv. Mater.*, 2011, **23**, 2957.
8 K. E. Kadler, C. Baldock, J. Bella and R. P. Boot-Handford, *J Cell Sci*, 2007, **120**, 1955.
9 (a) Y. Li and S. M. Yu, *Curr. Opin. Chem. Biol.*, 2013, **17**, 968; (b) D. E. Przybyla and J. Chmielewski, *Biochem.* 2010, **49**, 4411; (c) L. R. He and P. Theato, *Eur. Polym. J.*, 2013, **49**, 2986; (d) M. D. Shoulders and R. T. Raines, *Annu. Rev. Biochem.*, 2009, **78**, 929.
10 C. M. Rubert Pérez, A. Panitch and J. Chmielewski, *Macromol. biosci.*, 2011, **11**, 1426.
11 J. Josse and W. F. Harrington, *J. Mol. Biol.*, 1964, **9**, 269.
12 S. S. Raman, R. Parthasarathi, V. Subramanian and T. Ramasami, *J. Phys. Chem. B*, 2008, **112**, 1533.
13 (a) K. Mizuno, S. P. Boudko, J. Engel and H. P. Bachinger, *Biophys. J.*, 2010, **98**, 3004; (b) A. V. Persikov, Y. Xu and B. Brodsky, *Prot. Sci.*, 2004, **13**, 893.
14 (a) L. Klouda and A. G. Mikos, *Eur. J. Pharm. Biopharm.*, 2008, **68**, 34; (b) H. J. Moon, D. Y. Ko, M. H. Park, M. K. Joo and B. Jeong, *Chem. Soc. Re.v*, 2012, **41**, 4860.
15 (a) S. L. Bellis, *Biomater.*, 2011, **32**, 4205; (b) U. Hersel, C. Dahmen and H. Kessler, *Biomater.*, 2003, **24**, 4385.
16 C. G. Knight, L. F. Morton, A. R. Peachey, D. S. Tuckwell, R. W. Farndale and M. J. Barnes, *J. Biol. Chem.*, 2000, **275**, 35.

