Journal of Materials Chemistry B

COMMUNICATION

Biocompatible small peptide super-hydrogelators bearing carbazole functionalities

Adam D. Martin,*[a] Andrew B. Robinson,[a] Pall Thordarson*[a]

For the first time we have introduced carbazole capping groups onto two short peptide sequences, namely a diphenylalanine dipeptide and a glycine-diphenylalanine tripeptide, giving compounds 1 and 2, respectively. Both molecules form hydrogels at low concentrations, as low as 0.03% (w/v) for 1 – well within the range of supergelators. Both gelators are composed of small c.a. 2 nm thick molecular fibres, as elucidated by AFM and are non-cytotoxic towards HeLa cells at concentrations which are at or above their minimum gelation concentration.

Introduction

Biomimetic materials have recently attracted large amounts of interest due to their potential applications in the fields of drug delivery,[1,2] as cell scaffolds[3] and for tissue engineering.[4,5] Hydrogels are an important class of biomimetic materials, as they are able to act as mimics for the extracellular matrix, which promotes cell adhesion, differentiation, growth and proliferation.[6-8] Unlike polymer hydrogels, which are typically formed through the incorporation of water-soluble groups on a synthetic polymer backbone,[9,10] supramolecular hydrogels offer an advantage in that they are reversible in nature, owing to the non-covalent interactions that comprise the gel network. Peptide hydrogels are an important subset of supramolecular hydrogels which are promising materials for applications within the human body due to them being composed of naturally occurring amino acids.[11-13]

The variety of peptides that can be used for biological applications is almost limitless, however short peptides are attractive targets due to their ease of synthesis, the scalable nature of this synthesis and gelation ability. Numerous 9-fluorenylmethyloxycarbonyl (Fmoc)-capped dipeptides have been reported in the literature,[14-16] however perhaps the most well-known example is Fmoc-diphenylalanine (Fmoc-Phe-Phe).[17] This peptide has been shown to self-assemble into molecular fibres approximately 2 nm thick and while it has often been suggested that it would be useful for medical applications, it does appear to show some cytotoxicity *in vitro* at elevated concentrations.[18] Typically, diphenylalanine-based gelators require low pH in order to form a hydrogel, however the addition of a less hydrophobic glycine residue has been shown to raise the pH at which gelation occurs,[19] rendering the hydrogel more biocompatible. This has been observed for multiple capping groups.[20]

The selection of aromatic capping group also plays a significant role in the properties exhibited by the resultant hydrogel. For short peptides, an aromatic capping group is often required to drive gelation due to hydrophobic and π-stacking interactions. The most common capping group is Fmoc, however other capping groups are based upon different aromatic molecules such as anthracene,[21] naphthalene,[22] perylene[23] and pyrene.[24] These capping groups all provide the hydrophobic interactions necessary, and in the case of the last two, impart fluorescence upon the resultant gels. Heterocycles have also been employed as effective capping groups, with examples such as phenothiazine[25] and spiropyran reported.[26] Recently we synthesised an indole-capped dipeptide that displays extremely high values for its storage modulus, we theorised due to the hydrogen bonding potential of the indole moiety.[27]

Carbazole can be thought of as an analogue of the fluorene moiety that is present in the Fmoc capping group so widely used in peptide synthesis. Fluorene-capped dipeptides not containing the carbamate linkage (whereby peptides are connected via an amide linkage instead) have been synthesised and tend to be slightly less robust compared to their Fmoc analogues.[28,29] Herein we report the first examples of carbazole capped peptides, which were achieved through attachment of 9H-carbzole acetic acid to diphenylalanine (-Phe-Phe, Fig. 1a) and glycine-diphenylalanine (-Gly-Phe-Phe, Fig. 1b) amino acids.

Experimental

9*H*-carbazole acetic acid was synthesised according to a modified literature procedure.[30] –Phe-Phe and –Gly-Phe-Phe amino acid residues were synthesised using standard solid phase peptide synthesis techniques (see ESI for synthesis and characterisation data for capping group and capped peptides), with **1** and **2** being obtained in 48% and 49% yield, respectively. Both compounds were then purified using semi-preparative high performance liquid chromatography before further characterisation.

Results and Discussion

The gelation properties of both **1** and **2** were investigated after purification, initially using the vial inversion method. **1** forms hydrogels via a pH switch mechanism only, whereas **2** is able to form hydrogels either through pH switching, temperature switching in a 1 mM PBS buffer (pH 7.2) solution or through solvent switching, whereby an alkaline solution of **2** is diluted using DMEM, instantly forming a self-supporting hydrogel. It should be noted that for pH and solvent switching, sonication is used to initiallydisperse the gelator, forming a homogenous sol in all cases, before changing the solvent environment to result in gelation. **1** forms hydrogels at the remarkably low concentration of 0.03% (w/v), making this compound a supergelator and close to the lowest reported minimum gel concentration for a peptide hydrogel. In comparison to **1**, the minimum gel concentration for **2** is 0.1% (w/v), which is low, and indicates that both **1** and **2** have the ability to form ordered nanofibrous networks at extremely low concentrations. Given that the Fmoc analogues of **1** and **2** do not form hydrogels at such low concentrations (in fact, no gelation was reported for the Fmoc analogue of **2** using an enzymatic cleavage mechanism),[31] it can be inferred that these values observed are due to the selection of the carbazole capping group.
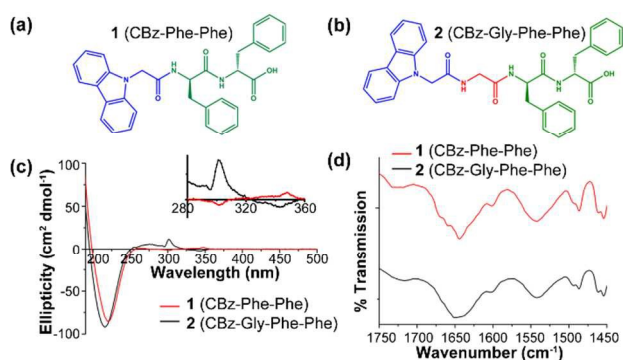

Fig. 1 Structure of carbazole capped peptides **1** (a) and **2** (b), CD spectra of hydrogels of **1** and **2** (c) and amide I and II region of the ATR-IR spectrum for 1% (w/v) hydrogels of **1** and (d) **2**.

The secondary structure of **1** and **2** was then probed using circular dichroism, with both hydrogels displaying a β-sheet structure (Fig. 1c). Due to difficulties in quantifying gel dilution, ellipticities per residue cannot be compared, however, the position of the minima in the CD spectrum are found at 221 and 216 nm for **1** and **2**, respectively, typical for hydrogels bearing the diphenylalanine motif. It is interesting to note that the hydrogels display features in the spectrum

from 300 – 350 nm that are opposite to each other but similar effects have been observed for peptides containing Gly-Phe-Phe.[32] ATR-IR spectra of hydrogels of **1** and **2** are almost identical, with only minor changes in the fine detail of some peaks (Fig. 1d). Hydrogels of **1** and **2** both contain peaks in the amide I (1600 – 1700 cm^{-1}) and amide II (1510 – 1580 cm^{-1}) regions, with the peak position in the amide II region confirming the β-sheet secondary structure obtained from CD measurements.
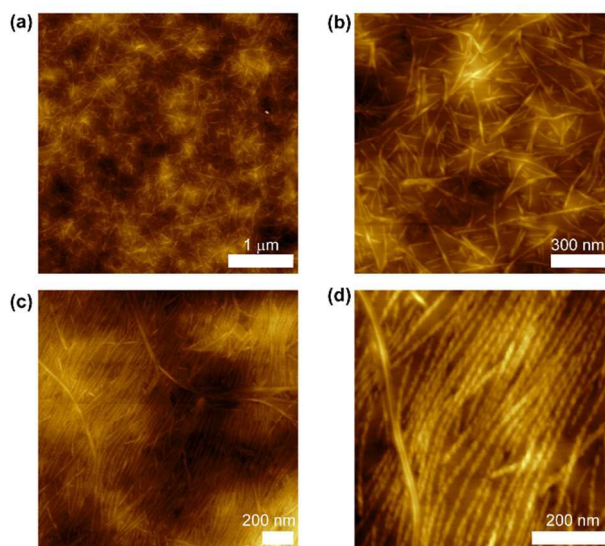

Fig. 2 AFM at different scan sizes, clearly showing fibre morphology of **1** (a, b) and **2** (c, d). Both hydrogel samples were prepared at a concentration of 0.1% (w/v) and imaged under ambient conditions.

In addition to investigating the secondary structure of hydrogels of **1** and **2**, the morphology of the gel network formed by **1** and **2** was also investigated. It was found that in both cases, small, molecular nanofibres comprise the gel network. For **1**, an average fibre size of 1.7 ± 0.3 nm was measured, which corresponds closely to the measured individual fibre size for Fmoc-Phe-Phe of approximately 2 nm. This implies that gels of **1** are composed of molecular fibres, much like the analogous Fmoc--Phe-Phe. For hydrogels of **2,** incorporation of a glycine residue serves to increase the fibre diameter to 2.2 ± 0.4 nm. This is smaller than the 20 nm fibre size reported for the gelator napthalanine-Gly--Phe-Phe,[20] and smaller than the 5 nm fibres measured in the Fmoc analogue of **2** (Fig. S16). This small fibre size persists even at higher concentrations and using different deposition methods (Fig. S6 and S12), indicating that the carbazole capping group has a propensity for forming small, molecular fibres.

Despite the similarities of **1** and **2** in terms of fibre size, their morphology is actually very different. It can be seen for hydrogels of **1,** the nanofibres observed are quite disjointed and not continuous (Fig. 2a). In this way, the system is more like a highly branched network of nano-fibrils. These fibrils do not possess a uniform length, and can be seen to intertwine and intersect with each other (Fig. 2b). For hydrogels of **2** however, this highly branched system of fibrils is replaced by fibres which are largely continuous, and although some fibre ends can be observed, the majority of the fibres imaged have lengths in the order of micrometers, Fig. 2c. Less branching is observed in this system; however fibres which intersect with each

other are still present. Perhaps the most striking difference though is the handedness that is clearly visible for these fibres. The vast majority of the fibres that can be seen in Fig. 2d are left handed, without any evidence for right handed fibres being present. The fact that this change in morphology is brought about by the addition of an achiral glycine residue in **2** is rather unexpected.
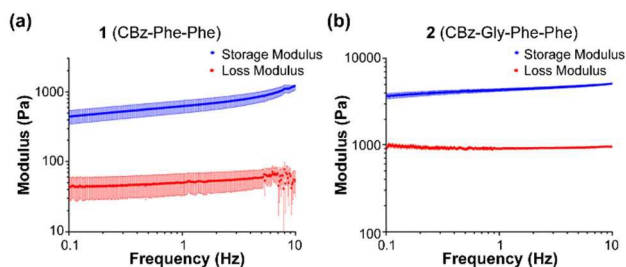

Fig. 3 Rheology of 1% (w/v) hydrogels of **1** (a) and **2** (b) performed at 25 °C. Error bars indicate standard deviation from repeat experiments (n = 3).

Rheology studies were then undertaken to investigate whether the difference in fibre morphology at the nanoscale resulted in a difference the macroscopic rheological properties for hydrogels of **1** and **2**. As can be seen from Fig. 3, this difference in nanoscale structure results in a large difference in gel strength, which is commonly approximated using the values obtained for the storage modulus G'. At 1% (w/v) and both gels exhibit linear behaviour over the frequencies measured. Hydrogels of **1** display a storage modulus of 0.5 – 0.7 kPa, compared to 3 kPa for hydrogels of **2**. This represents a greater than 4-fold increase in gel strength due to the incorporation of a glycine residue into the peptide structure. Rheology was also performed at the minimum gel concentrations of both hydrogels (Fig. S5 and S11). In this case, hydrogels of **1** have a storage modulus of 150 Pa, compared to 1 kPa for hydrogels to **2**. This confirms that even at low concentrations, hydrogels of **2** are stiffer than those of **1**. The weak nature of hydrogels of **1** is apparent from viewing the nanoscale structure – the discontinuous nature of the fibres, resulting in fibril formation coupled with the highly branched nature of these fibrils. These discontinuous fibrils mean that the persistence length (essentially the length of aligned segments within a fibre) for these hydrogels is low, and the storage modulus G' has been shown to be dependent on the square of persistence length.[33] This helps explain why the storage modulus values for hydrogels of **2** are so much higher, because even though the fibre size is still small, the persistence length of these fibres is much larger than for hydrogels of **1**.

The size of the fibres observed for hydrogels of **1** and **2**, coupled with their relatively low values for G' makes these materials excellent candidates for extracellular matrix mimics. To this end, preliminary cytotoxicity studies were undertaken using a HeLa cell line. It can be seen that both **1** and **2** are well tolerated up to a concentration of 0.1% (w/v). It should be noted that for both gelators, this is at or above their minimum gel concentration. Cytotoxicity results were similar over 24 and 48 hours (Fig. S15), suggesting that the cell death observed may be due to initial contact cytotoxicity, and is not a sustained process. Images of cells seeded on top of hydrogels of **1** and **2** show some cell spreading and proliferation (Figure S14), however the opacity of the

hydrogels prevented imaging at higher concentrations of gelator. It is also evident from Fig. 4 that cell viabilities are very similar for both **1** and **2**, meaning that it is most likely the selection of capping group, not the alteration of an amino acid residue that is responsible for any cytotoxic effects observed. Based upon the data in Figure 4, we can estimate IC_{50} values for **1** and **2** of 0.18 and 0.10% (w/v) respectively. In both cases, this value is at or above than the minimum gel concentration for these peptides. This makes both peptide gelators good candidates for further study employing different cell lines and as potential extracellular matrix (ECM) mimics or drug delivery vectors.
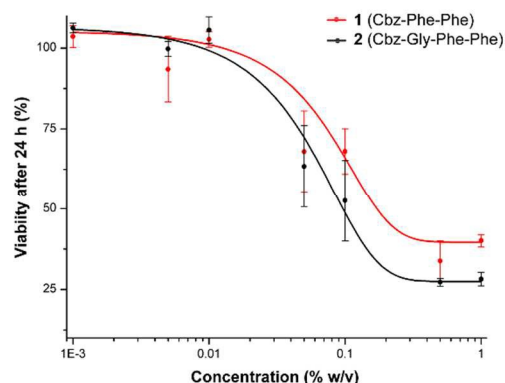

Fig. 4 Cytotoxicity studies of **1** and **2**, performed on HeLa cells with n = 3, over a time period of 24 h.

Conclusions

In conclusion, we have synthesised two novel peptides bearing for the first time a heterocycliccarbazole capping group. These short peptides differ by a single amino acid residue, and form self-supporting hydrogels at very low concentrations, and in the case of **1**, concentrations which make it a supergelator. Both peptides exhibit a β-sheet secondary structure and form nanofibres that are approximately 2 nm in diameter. Fibres of **1** are more discontinuous and have a high proportion of junctions, resulting in a soft gel, which has been confirmed by rheology. In contrast, **2** displays longer, left-handed nanofibres. Cytotoxicity studies on HeLa cells confirm that the gels are well tolerated up to 0.1% (w/v), which for both peptides is at or above their minimum gel concentration –in the case of **1**, almost an order of magnitude above its minimum gel concentration.

Notes and references

[a]School of Chemistry, The Australian Centre for Nanomedicine and the ARC Centre of Excellence for Convergent Bio-Nano Science and Technology, The University of New South Wales, Sydney, 2052, NSW, Australia.

Email: adam.martin2@unsw.edu.au, p.thordarson@unsw.edu.au; Fax: +61 (0)2 9385 6141; Tel: +61 (0)2 9385 4478.

1   M. C. Branco and J. P. Schneider, *ActaBiomater.*, 2009, **5**, 817.
2   A. Altunbas and D. J. Pochan, *Top. Curr. Chem.*, 2011, **310**, 135.
3   H. Wang, Y. Li, Y. Zuo, J. Li, S. Ma and L. Cheng, *Biomaterials*, 2007, **28**, 3338.
4   J. L. Drury and D. J. Mooney, *Biomaterials*, 2003, **24**, 4337.
5   S. Van Vlierberghe, P. Dubruel and E. Schacht, *Biomacromolecules*, 2011, **12**, 1387.
6   M. P. Lutolf and J. A. Hubbell, *Nat. Biotechnol.*, 2005, **23**, 47.
7   K. Y. Lee and D. J. Mooney, *Chem. Rev.*, 2001, **101**, 1869.
8   M. M. Stevens and J. H. George, *Science*, 2005, **310**, 1135.
9   T. Aida, E. W. Meijer and S. I. Stupp, *Science*, 2012, **335**, 813.
10   S. Chaterji, I. K. Kwon and K. Park, *Prog. Polym. Sci.*, 2007, **32**, 1083.
11   E. K. Johnson, D. J. Adams and P. J. Cameron, *J. Mater. Chem.*, 2011, **21**, 2024.
12   R. G. Weiss, *J. Am. Chem. Soc.*, 2014, **136**, 7519.
13   A. Dasgupta, J. H. Mondal and D. Das, *RSC Adv.*, 2013, **3**, 9117.
14   N. Javid, S. Roy, M. Zelzer, Z. Yang, J. Sefcik and R. V. Ulijn, *Biomacromolecules*, 2013, **14**, 4368.
15   Z. Yang, H. Gu, D. Fu, P. Gao, J. K. Lam and B. Xu, *Adv. Mater.*, 2004, **16**, 1440.
16   Y. Zhang, H. Gu, Z. Yang and B. Xu, *J. Am. Chem. Soc.*, 2003, **125**, 13680.
17   M. Reches and E. Gazit, *Israel J. Chem.*, 2005, **45**, 363.
18   W. T. Truong, Y. Su, D. Gloria, P. Braet and P. Thordarson, *Biomater. Sci.*, 2015, **3**, 298-307.
19   M. He, J. Li, S. Tan, R. Wang and Y. Zhang, *J. Am. Chem. Soc.*, 2013, **135**, 18718.
20   H. Wang, Z. Wang, X. Yi, J. Long, J. Liu and Z. Yang, *Chem. Commun.*, 2011, **47**, 955.
21   K. Heinze and K. Hempel, *Chem. Eur. J.*, 2009, **15**, 1346.
22   L. Chen, K. Morris, A. Laybourn, D. Elias, M. R. Hicks, A. Rodger, L. Serpell and D. J. Adams, *Langmuir*, 2010, **26**, 5232.
23   A. S. Weingarten, R. V. Kazantsev, L. C. Palmer, M. McClendon, A. R. Koltonow, A. P. S. Samuel, D. J. Kiebala, M. R. Wasielewski and S. I. Stupp, *Nat. Chem.*, 2014, **6**, 964.
24   Y. Zhang, Z. M. Yang, F. Yuan, H. W. Gu, P. Gao and B. Xu, *J. Am. Chem. Soc.*, 2004, **126**, 15028.
25   C. Ou, J. Zhang, X. Zhang, Z. Yang and M. Chen, *Chem. Commun.*, 2013, **49**, 1853.
26   Z. J. Qiu, H. T. Yu, J. B. Li, Y. Wang and Y. Zhang, *Chem. Commun.*, 2009, **45**, 3342.
27   A. D. Martin, A. B. Robinson, A. F. Mason, J. P. Wojciechowski and P. Thordarson, *Chem. Commun.*, 2014, **50**, 15541.
28   S. Fleming, P. W. J. M. Frederix, I. R. Sassela, N. T. Hunt, R. V. Ulijn and T. Tuttle, *Langmuir*, 2013, **29**, 9510.
29   S. Fleming, S. Debnath, P. W. J. M. Frederix, T. Tuttle and R. V. Ulijn, *Chem. Commun.*, 2013, **49**, 10587.
30   M. Milen, A. Grun, E. Balint, A. Dansco and G. Keglevich, *Synthetic Commun.*, 2010, **40**, 2291.
31   S. Toledano, R. J. Williams, V. Jayawarna and R. V. Ulijn, *J. Am. Chem. Soc.*, 2006, **128**, 1070.
32   Y. Tian, H. Wang, Y. Liu, L. Mao, W. Chen, Z. Zhu, W. Liu, W. Zheng, Y. Zhao, D. Kong, Z. Yang, W. Zhang, Y. Shao and X. Jiang, *Nano Lett.*, 2014, **14**, 1439.
33   P. J. H. Kouwer, M. Koepf, V. A. A. Le Sage, M. Jaspers, A. M. van Buul, Z. H. Eksteen-Akeroyd, T. Woltinge, E. Scwartz, H. J. Kitto, R. Hoogenboom, S. J. Picken, R. J. M. Nolte and A. E. Rowan, *Nature*, 2013, **493**, 651.

Two novel short peptides bearing a novel carbazole capping group form gels at concentrations as low as 0.03% w/v and are biocompatible at or above their minimum gel concentrations.