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Phenylalanine-containing cyclic dipeptides – the lowest molecular weight hydrogelators based on unmodified proteinogenic amino acids†

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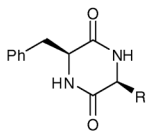
Cyclic dipeptides (diketopiperazines – DKPs) that are based on the proteinogenic amino acid phenylalanine in combination with serine, cysteine, glutamate, histidine and lysine are described as simple and remarkable low molecular weight hydrogelators. Blends of selected DKPs show remarkable pH-dependent properties and can be applied as easy to tune materials in drug delivery.



1 (R=H), cyclo(L-Phe-Gly), MW = 204 g/mol
2 (R=CH_2-OH), cyclo(L-Phe-L-Ser), MW = 234 g/mol
3 (R=CH_2-SH), cyclo(L-Phe-L-Cys), MW = 250 g/mol
4 (R=CH_2-CH_2-CO_2H), cyclo(L-Phe-L-Glu), MW = 276 g/mol
5 (R=CH_2-(4-imidazolyl), cyclo(L-Phe-L-His), MW = 284 g/mol
6 (R=$(CH_2)_4$-NH_2), cyclo(L-Phe-L-Lys), MW = 275 g/mol

Fig. 1 Investigated cyclic dipeptides.

Physical molecular hydrogels based on low molecular weight gelators (LMWGs) have become an important class of materials due to their versatile applications as innovative soft materials in injection based drug delivery and tissue engineering.[1] The gel-resulting self-assembly process of LMWGs is initiated by non-covalent forces such as hydrogen bonding, π-stacking or van der Waals interactions. Above all, amino acids and small peptides are popular LMWGs since they are easy to vary and often show promising biocompatibility.[2,3] However, intermolecular hydrogen bonding networks between amide bonds alone, in particular in water, are in general not sufficient to make a small peptide or amino acid an efficient hydrogelator. Thus, functionalization of the peptide *N*- or *C*-terminus with protecting groups capable of excessive π-stacking interactions is necessary. Low molecular weight peptide based hydrogelators which only contain unmodified proteinogenic amino acids are rare.[4] In our search for the smallest amino acid based units that can form higher ordered structures in an aqueous environment,[5] we herein want to present cyclic dipeptides (diketopiperazines – DKPs) as representative examples for remarkable small LMWGs that self-assemble into nanofibers and form stable, self-healing hydrogels.[6,7]

Due to the rigidity of the 6-membered ring, we predicted that the self-assembly of DKPs into higher ordered structures *via* cooperative intermolecular hydrogen bonding should be entropically much more favourable than it would be for comparable open-chained di- or oligopeptides. Therefore, we synthesized a small set of DKPs containing the lipophilic amino acid phenylalanine and a variety of hydrophilic amino acids, in particular glycine (**1**), serine (**2**), cysteine (**3**), glutamate (**4**), histidine (**5**) and lysine (**6**) (Fig. 1). In an initial experiment we were intended to verify the principle hydrogelation ability of cyclic dipeptides **1–6**. Thus, we heated an aqueous suspension containing 4 wt% of each DKP until the solid completely dissolved. Subsequently, the saturated peptide-containing solution was cooled to rt or even lower. To our great delight, each DKP **1–6** formed stable hydrogels under these conditions (Fig. 2).

DKPs **1–3** and **5** form opaque hydrogels. Glutamate-containing DKP **4** yields a transparent hydrogel (at pH 6.0 in phosphate buffer). The lysine-containing DKP **6** also yields a transparent hydrogel. To further elucidate their thermal stability, we examined the sol–gel transition temperature of these hydrogelators systematically as a function of DKP-concentration (ESI,† Fig. S1A). Cyclo(L-Phe-L-Cys) **3** was the most effective hydrogelator since it formed a stable hydrogel down to a remarkably low concentration of only 0.25 wt%. It shows a notable high sol–gel transition temperature of up to 100 °C at 1 wt%. In contrast, the corresponding serine-containing DKP **2** turned out to be the least efficient hydrogelator since high peptide loadings (at least 4 wt%) are needed for a hydrogelation with a low sol–gel transition

Fig. 2 Macroscopic appearance of DKP-based hydrogels; (A) cyclo(L-Phe-Gly) **1**. (B) Cyclo(L-Phe-L-Ser) **2**. (C) Cyclo(L-Phe-L-Cys) **3**. (D) Cyclo(L-Phe-L-Glu) in phosphate buffer at pH 6.0. (E) Cyclo(L-Phe-L-His). (F) Cyclo(L-Phe-L-Lys).

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† Electronic supplementary information (ESI) available: Synthetic procedures, spectroscopic data as well as ^{1}H and ^{13}C spectra of DKPs **1–6**, rheological measurements and concentration dependent sol–gel transition temperatures of **1–6** and blends of **4** and **6**. See DOI: 10.1039/c3cc44110e

temperature of 12 °C. In general, all DKPs, except **2**, form stable hydrogels at rt with a low concentration of 2 wt%. Cyclo(L-Phe-Gly) **1** which bears no hydrophilic functional group forms stable hydrogels down to a concentration of 1 wt%. To the best of our knowledge, **1** is the lowest molecular weight peptide-based hydrogelator containing only proteinogenic amino acids that has been described so far.[8,9]

Next, we were interested in the shape and morphology of the hydrogels on a nanoscale. Therefore, we prepared xerogels of the corresponding hydrogels through freeze drying and performed scanning electron microscopy (SEM) (Fig. 3). Xerogel morphology of **1** showed porous, but highly ordered planes (Fig. 3A and B). Each plane is woven by knotted nanofibers. DKP **2** also consists of porous planes (Fig. 3C and D) which contain entangled nanofibers and partly microcrystalline domains. DKP **3** is constructed by undefined nanosheets (Fig. 3E and F) while the glutamate-containing DKP **4** (Fig. 3E and F) forms a defined porous network at pH 6.0 (in phosphate buffer).

DKPs **5** and **6** form dense lamellar sheets with bundled nanofiber connections between the sheets (Fig. 3I–L). The viscoelastic properties of the hydrogels were investigated by rheological strain sweep experiments (ESI,† Fig. S2–S7A). The values of the storage modulus G' are in the remarkable range of 10^3–10^6 Pa. A strong age-dependent storage modulus was observed for the lysine containing hydrogel **6** which increases from 697 Pa for a freshly prepared gel sample to 146 kPa for a gel sample aged for 20 h prior to the rheological measurement. On the other hand, the freshly prepared sample showed the highest mechanical stability with a high critical strain level of 10% deformation. Further time sweep experiments showed a remarkable increase of G' upon aging for all DKPs (ESI,† Fig. S2–S7D). In agreement with comparable hydrogels, the loss moduli (G'') were found to be at least one order of magnitude lower than G' indicating that the hydrogels described herein are true physical hydrogels and not viscoelastic fluids.[10] Frequency sweep experiments of all hydrogels showed a very weak dependency on the angular frequency suggesting that the gel matrices have excellent tolerance towards external forces (ESI,† Fig. S2–S7B). Furthermore, most of the hydrogels (except of **2**) showed a significant thixotropic behaviour and thus have self-healing properties (ESI,† Fig. S2–S7C). We further asked ourselves whether we can tune the mechanical and morphological properties of the hydrogel by mixing two or more DKPs. Due to recent reports regarding tuneable two-component hydrogels based on the interaction between diamines and carboxylic acids,[11,12] we studied the gelation behaviour of a 1 : 1 mixture between the glutamate containing DKP **4** and the lysine containing DKP **6**.

We initially investigated the sol–gel transition temperature of the blended hydrogel and found remarkable pH-dependency. While pure **4** only formed stable hydrogels at pH 6.0 and pure **6** only formed stable hydrogels in basic solution (pH 8–11), the blend formed a clear hydrogel within a remarkably widespread pH-range between pH 2 and pH 11. The sol–gel temperature reaches a maximum at pH 2 (79 °C) and a minimum at neutral pH-values (27 °C, Fig. 4B). The viscoelastic properties showed significant pH-dependency as well, which was inverse to the sol–gel temperatures with a maximum G' value

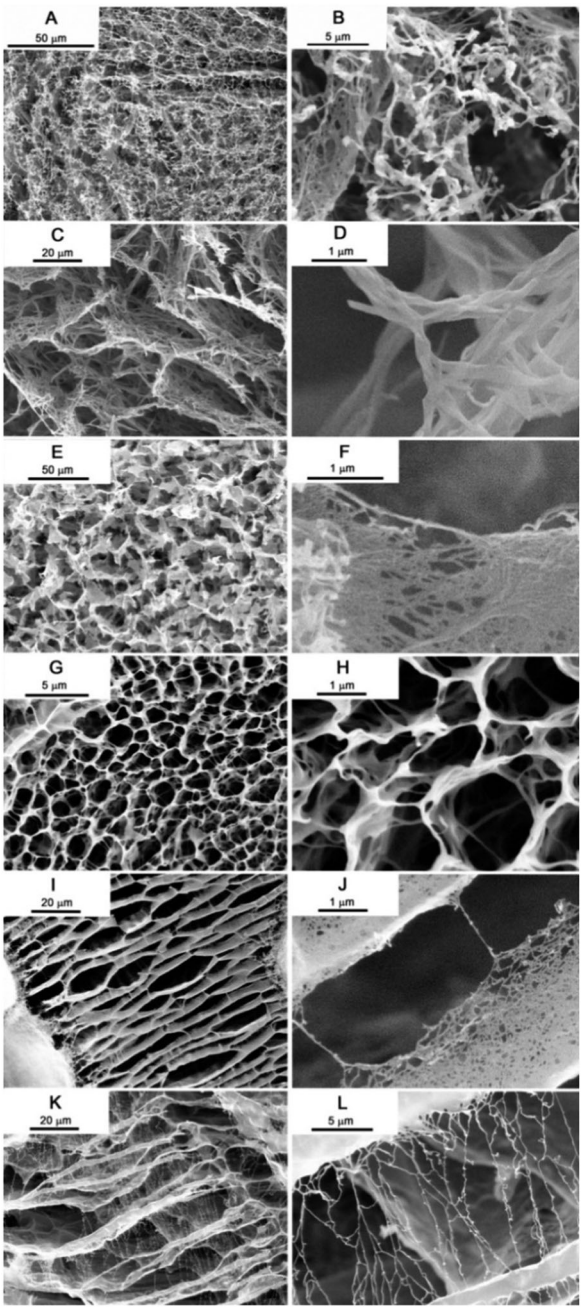

Fig. 3 SEM images of xerogels. Unless otherwise stated, gelation was performed in an unbuffered aqueous solution (A and B) cyclo(L-Phe-Gly) **1** at pH = 7. (C and D) Cyclo(L-Phe-L-Ser) **2** at pH = 7. (E and F) Cyclo(L-Phe-L-Cys) **3** at pH = 7. (G and H) Cyclo(L-Phe-L-Glu) **4** at pH = 6.0 (phosphate buffer). (I and J) Cyclo(L-Phe-L-His) **5** at pH = 8. (K and L) Cyclo(L-Phe-L-Lys) **6** at pH = 10–10.5.

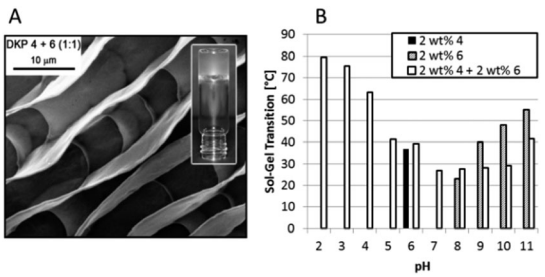

Fig. 4 (A) SEM image of a freeze-dried xerogel (2 wt% **4** and 2 wt% **6** at pH 7). (B) pH-dependent sol–gel temperature of a 1 : 1 blend of **4** and **6** (2 wt% each) compared to pure DKPs (2 wt%).

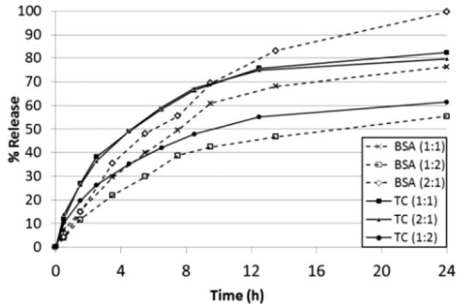

Fig. 5 Drug release experiment with BSA (dotted lines) and tetracycline (continuous lines) in different blend hydrogels of DKPs **4** and **6**. (DKP **4** : DKP **6**).

at pH 7 (ESI,† Fig. S8A). However, the fastest regeneration is observed at pH 10 (ESI,† Fig. S8B). A SEM image of a freeze dried sample at pH 7 showed a so far unseen morphological shape with a highly ordered lamellar structure (Fig. 4A). Xerogel morphologies for other pH values are shown in the ESI† (Fig. S9).

Finally, we were interested in an application of our newly found hydrogel blends in a drug release experiment. We particular asked ourselves whether we can modulate the drug releasing properties of the hydrogel by simply varying the DKP ratio. Thus 1 : 1, 1 : 2 and 2 : 1 mixtures of **4** and **6** were investigated in the release of (1) tetracycline (TC) as a lipophilic small molecule and (2) bovine serum albumin (BSA) as a highly charged polypeptide as model substrates (Fig. 5).[13]

For both substrates a significant difference in drug release was observed. While nearly all BSA was released after 24 h for a 2 : 1 mixture of **4** and **6**, only 55% was released with the 1 : 2 mixture. The hydrogel containing a 1 : 1 mixture of **4** and **6** lies in-between (75%). For TC the fastest release was observed for the 1 : 1 and the 2 : 1 mixtures (82% and 80%) while the 1 : 2 mixture showed a significantly delayed release (62%).

In summary, we herein present the remarkable hydrogelation properties of phenylalanine-containing cyclic dipeptides (diketopiperazines – DKPs) which only contain proteinogenic amino acids as building blocks. To the best of our knowledge these structures are the smallest peptide-based hydrogelators that have been described so far. Despite their simplicity and the low molecular weight of their monomeric building blocks, the so formed hydrogels are remarkably stable and self-healing. We could further demonstrate that a blend of two well-chosen DKPs shows novel pH-dependent mechanical properties and can be used as soft materials for delayed drug release.

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