RSC Advances

COMMUNICATION

View Article Online
View Journal | View Issue

D-amino acid doping peptide hydrogel for the production of a cell colony†

Cite this: *RSC Adv.*, 2014, **4**, 9229

Received 19th November 2013
Accepted 23rd January 2014

DOI: 10.1039/c3ra46847j

www.rsc.org/advances

Caiwen Ou,‡[a] Jianwu Zhang,‡[a] Yang Shi,[b] Zhongyan Wang,[c] Ling Wang,[c] Zhimou Yang*[b] and Minsheng Chen*[a]

We report on a rationally designed D-amino acid doping peptide that can form hydrogels under neutral conditions and can be applied to form a cell colony of HeLa cells.

Peptide-based hydrogels[1] have been demonstrated to be promising biomaterials for controlled release of bioactive molecules,[2,3] cell culture,[4] and regenerative medicine.[5,6] In order to homogeneously encapsulate cells or proteins in hydrogels, biocompatible methodologies to trigger the formation of hydrogels hold advantages. Several biocompatible methods to form peptide-based hydrogels have been reported so far, including ionic strength change,[7] enzyme triggeration,[8] light irradiation,[9] disulfide bond reduction,[10] and ligand–receptor interaction.[11] Among these methods, the one of ionic strength change is probably the most frequently used. It has been applied to form many hydrogels of peptides such as peptide amphiphiles,[3] β-hairpin peptides,[12] RADA16 peptide and its analogs,[13] Q11 peptide and its analogs,[6,14] and EFK8 peptide and its analogs.[15] Based on the reported literature, most of these peptides can form clear solutions in pure water solution at neutral or near neutral condition. The hydrogels can then form upon mixing these pure water solutions of peptides with other higher ionic strength buffer solutions, such as phosphate buffer saline (PBS) and cell culture medium. However, EFK8 can only form pure water solution at acidic condition and then form homogeneous gels upon mixing with buffer solution also at acidic condition (usually pH around 3).[16]

Among the reported pure peptide-based (not those capped with aromatic capping groups) hydrogelators, EFK8 is one of the simplest and mostly investigated ones. However, it can only form hydrogels at acidic condition, which hinders its applications for such as cell culture. As shown in Fig. 1A, the EFK8 peptide exhibits the property of one side aromatic (phenylalanine (F) side chain) and the other side of alternative positive (lysine (K) side chain) and negative (glutamic acid (E) side chain) charges. The aromatic interaction between phenyl rings on Fs and positive–negative charge interaction between Es and Ks help to extend the supramolecular chain to form networks of nanofibers for hydrogelation. Due to the strong aggregation property in pure water solutions at neutral pH, it can only form clear solutions in acidic condition. Many efforts have been paid to develop EFK8 peptide derivatives that can form hydrogels at neutral condition. For example, Zhang and co-workers rationally altered the hydrophobic side-chains, the charged side-chains, and the number of repeats to create a self-assembling oligopeptide sequence that at pH of 7 remained viscous in the absence of salt but gelled in the presence of physiological salt concentrations.[17] Nilsson *et al.* changed the amino acid of E on EFK8 to glutamine (Q) to reduce the charge interaction. They also replaced F with less aromatic and non-polar amino acids such as alanine (A) and valine (V) to reduce the aromatic interaction.[18] These rational designs can reduce the aggregation property of the peptides in pure water solutions at neutral condition. Ulijn and co-workers recently used the enzyme of thermolysin to trigger the reverse-hydrolysis of the tetrapeptide of FEFK and subsequent gelation of ionic-complementary peptides at neutral condition.[19] These efforts had led to the formation of hydrogels of peptides at neutral condition.

In this study, we reported on our strategy to form hydrogels of EFK8 peptide derivatives at neutral condition by replacement of F with ^{D}F and the introduction of a hydrophilic RGD tripeptide. We realized that the orientation of aromatic rings on F was similar and peptide side chains were on the same planar for EFK8 peptide (Fig. 1C and E). If replacing F with ^{D}F, it might decrease the strong aggregation potency of the peptide in pure

[a]*Department of Cardiology, Zhujiang Hospital of Southern Medical University, Guangzhou 510280, P. R. China. E-mail: gzminsheng@vip.163.com*

[b]*State Key Laboratory of Medicinal Chemical Biology and College of Life Sciences, Nankai University, Collaborative Innovation Center of Chemical Science and Engineering (Tianjin), Tianjin 300071, P. R. China. E-mail: yangzm@nankai.edu.cn*

[c]*College of Pharmacy and Tianjin Key Laboratory of Molecular Drug Design, Nankai University, Tianjin 300071, P. R. China*

† Electronic supplementary information (ESI) available: Hydrogel preparation, rheology, CD spectra, FTIR spectra, *in vivo* stability study, and detail experimental procedure. See DOI: 10.1039/c3ra46847j

‡ The authors pay equal contributions to this work.

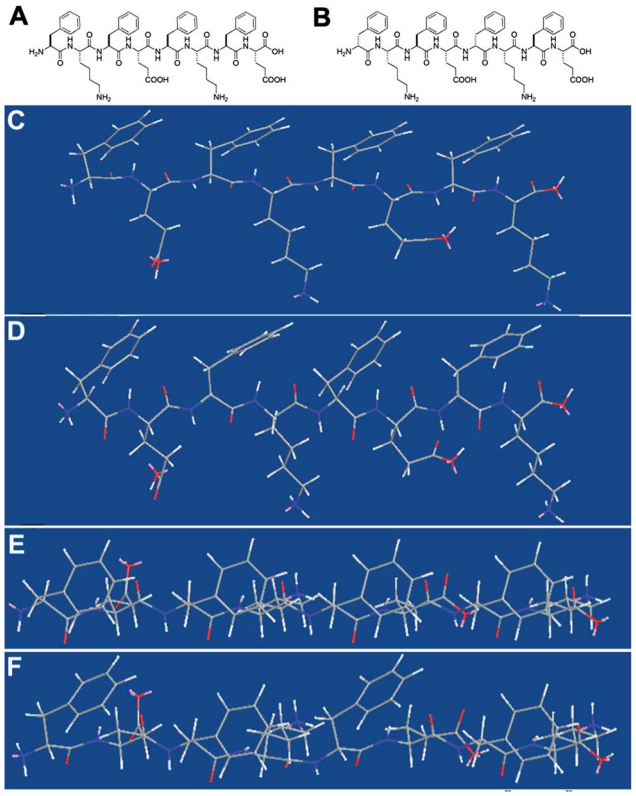

Fig. 1 Chemical structures of (A) FEFKFEFK and (B) ^{D}FEFK^{D}FEFK, molecular models of peptides at minimized energy condition by running MM2 in Chem3D (stop condition: minimum RMS = 0.05, (C) and (E): FEFKFEFK, (D) and (F): ^{D}FEFK^{D}FEFK) (FEFKFEFK: the orientation of aromatic rings on F is similar and peptide side chains are on the same planar. ^{D}FEFK^{D}FEFK: two aromatic rings on ^{D}F are not on the same planar with other peptide side chains and the orientation of aromatic rings on ^{D}F is different to that on F).

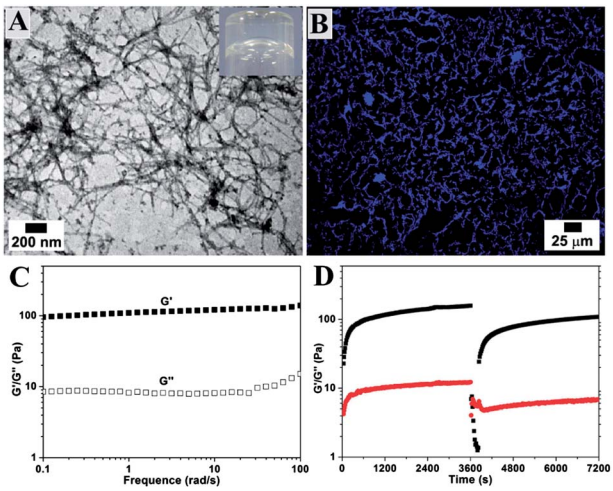

Fig. 2 (A) TEM and the optical image of the gel (1.0 wt%), (B) the confocal image of the gel stained with Nile blue, (C) the dynamic frequency sweep of the gel at the strain value of 1%, and (D) recovery property of the gel (the gel was measured under dynamic time sweep for 3600 seconds (strain = 1% and frequency = 1 rad s^{-1}). It was then subjected to a large strain of 100% for 300 seconds, following by measuring the recovery property at the strain value of 1% and frequency value of 1 rad s^{-1}).

water solution at neutral condition (in this study, at pH 7.4) because this strategy would change the original orientation of aromatic rings (Fig. 1D and F: two aromatic rings on ^{D}F were not on the same planar with other peptide side chains and the orientation of aromatic rings on ^{D}F was different to that on F). We firstly designed and synthesized a peptide of ^{D}FEFK^{D}FEFK. However, it could not form clear solution in pure water at pH of 7.4 but dissolved only at acidic condition. We therefore synthesized the peptide of ^{D}FEFK^{D}FEFKYRGD. The hydrophilic and bioactive peptide of RGD could further increase the solubility of the resulting peptide, and the Y could be labelled with radioactive ^{125}I that would facilitate testing the *in vivo* stability of resulting hydrogels.

We found that ^{D}FEFK^{D}FEFKYRGD peptide could form clear solutions in pure water at pH of 7.4. Hydrogels were then formed upon mixing the viscous solution with equal volume of 2× PBS (Fig. 2A, insrt). The minimum concentration needed for gelation for the peptide was about 0.15 wt%. As we reported before, the peptide FEFKFEFKGRGD could only form suspension in PBS at neutral conditions.[20] These observations indicated that the aggregation potency of peptides in pure water at neutral condition could be decreased by both D-amino acid replacement and the attachment of a hydrophilic peptide. Peptides by replacing other F with ^{D}F (^{D}FEFKFE^{D}FKYRGD and FEFK^{D}FE^{D}FKYRGD) could not form clear solutions in pure water solution at pH 7.4 (Fig. S7†). The mechanisms for these observations were needed to be studied in near future by us.

Transmission electron microscopy (TEM) image revealed filamentous structures with the size of about 40 nm in the hydrogel (Fig. 2A). Confocal image of the gel indicated a non-continuous network of small aggregates with size of about 10 μm (Fig. 2B). Rheological measurement with the mode of dynamic frequency sweep in Fig. 2C showed that the elasticity (G′) of the gel was about 100 Pa at the frequency range from 0.1 to 100 rad s^{-1}. The gel formed after 1 h was subjected to a large amplified of strain (100%) for 300 seconds. The recovery property was then recorded at the strain of 1%. The results in Fig. 2D exhibited a fast recovery property of the hydrogel and the G′ value re-gained 90% of its original G′ value within 1 h. The good recovery property of the gel correlated well with its confocal image in Fig. 2B because it was reported that gels with non-continuous networks in confocal images would possess better recovery property than those with continuous networks.[21]

Since hydrogels of the peptide could form at pH of 7.4 in PBS and complete cell culture medium (Dulbecco's Modified Eagle's Medium (DMEM) with 10% of FBS, Fig. S10†), we tested the possibility of the hydrogel for 3D cell culture. We mixed pure water solutions containing 2 wt% of the peptide with equal volume of complete cell culture medium containing 3×10^6 per mL of HeLa cells, and the cell-gel constructs could form within 5 minutes (final peptide concentration = 1.0 wt% and cell density was 1.5×10^6 per mL). As shown in Fig. 3A, the live–dead assay of cell-gel construct at day 1 indicated that most of cells were alive, which was showed in green dots. The results in Fig. 3A–D showed that the HeLa cells kept dividing but did not

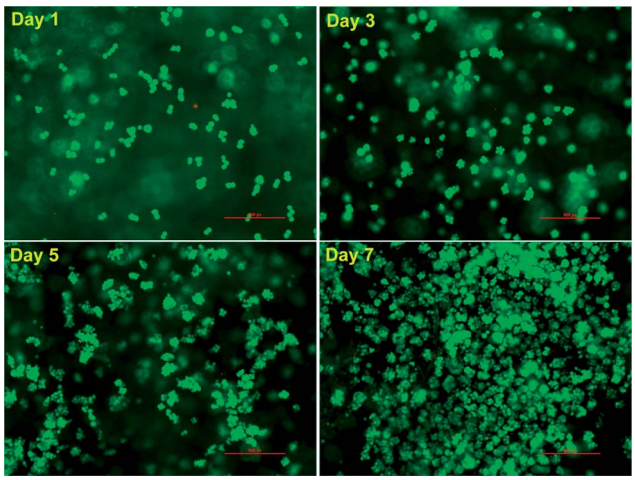

Fig. 3 Live–dead assay (live cells are in green and dead ones are in red) and fluorescence microscopy images of HeLa cells cultured in gel (1.0 wt%) at different time points (10×).

spread and remained as round shape during the 7 days culture period. It was very interesting that the cell number kept increasing during the 7 days culture period and HeLa cells grew into cell colonies. Many reports had demonstrated that cancer cell colony cultured on 3D scaffolds retained significantly more oncological character than those cultured on conventional 2D substrates.[22] Therefore, HeLa cell colony in our peptide hydrogel might be used as *in vitro* tumor model for the evaluation of activity of anti-cancer drugs or drug candidates.

The cell proliferation rate of HeLa cells in hydrogels with different concentrations of peptide was also determined (Fig. S11†). The results indicated that cell-gel constructs with three concentrations of the peptide (0.3, 0.5, and 1 wt%) behaved similar in the cell proliferation rate. For cell-gel constructs at peptide concentration of 1.0 wt%, the OD value was 144%, 363%, 620% higher at day 3, day 5, and day 7 than that at day 1, respectively. These results suggested that our peptide hydrogels could assist the efficient division of HeLa cells, which was consistent with the results shown in Fig. 3A–D.

We also evaluated the *in vivo* stability of peptide hydrogels with different peptide concentrations. The peptide with radioactive iodination (^{D}FEFK^{D}FEFK^{I}*YRGD) was incorporated into the gels and the resulting gels were then injected into the subcutaneous space of mice. The *in vivo* stability of the hydrogels was determined by measuring the radioactive signals from the injection sites. The results indicated that less than 10% of peptides remained in the injection sites after 24 h (Fig. S13 and S14†) even for the hydrogel containing 1.0 wt% of the peptide. For hydrogels at lower concentrations of peptides, less than 2% of radioactive signals were detected at 24 h time point (Fig. S13 and S14†). These results suggested a fast clearance and rapid degradability of the hydrogels *in vivo*. The fast clearance of hydrogels were probably due the injection-caused fragmentation of peptide hydrogels and the existence of digestion enzymes.

In summary, we reported on a rationally designed peptide that could form hydrogels at neutral condition. The hydrogels could be applied for the production of cell colony of HeLa cells. Many literature have indicated that *in vitro* cultured cancer cells with colony morphology behaved more similar to cancer cells *in vivo* (in tumors) than individual cancer cells obtained by conventional 2D culture method. Materials that can be used to produce cancer cell colonies are also important for the evaluation of anti-cancer drugs. Though our hydrogel showed a fast clearance *in vivo*, the hydrogel might be very promising for the production of colonies of different cells *in vitro*. The production of cell colony of different cells will be promising for regenerative medicine and cell therapy. This possibility will be testified by us in near future.

Acknowledgements

This work is supported by NSFC (51003049 and 31271053) and Tianjin MSTC (12JCYBJC11300).

Notes and references

1 J. W. Steed, *Chem. Commun.*, 2011, **47**, 1379; L. A. Estroff and A. D. Hamilton, *Chem. Rev.*, 2004, **104**, 1201; J. H. Collier, J. S. Rudra, J. Z. Gasiorowski and J. P. Jung, *Chem. Soc. Rev.*, 2010, **39**, 3413; Y. Gao, F. Zhao, Q. Wang, Y. Zhang and B. Xu, *Chem. Soc. Rev.*, 2010, **39**, 3425; R. V. Ulijn and A. M. Smith, *Chem. Soc. Rev.*, 2008, **37**, 664; H. Wang, Z. Yang and D. J. Adams, *Mater. Today*, 2012, **15**, 500; H. Wang and Z. Yang, *Nanoscale*, 2012, **4**, 5259; Y. Li, Y. Ding, M. Qin, Y. Cao and W. Wang, *Chem. Commun.*, 2013, **49**, 8653.

2 X. D. Xu, L. A. Liang, C. S. Chen, B. Lu, N. L. Wang, F. G. Jiang, X. Z. Zhang and R. X. Zhuo, *ACS Appl. Mater. Interfaces*, 2010, **2**, 2663; A. G. Cheetham, P. Zhang, Y.-A. Lin, L. L. Lock and H. Cui, *J. Am. Chem. Soc.*, 2013, **135**, 2907; J. Li, Y. Kuang, Y. Gao, X. Du, J. Shi and B. Xu, *J. Am. Chem. Soc.*, 2012, **135**, 542; X. Li, J. Li, Y. Gao, Y. Kuang, J. Shi and B. Xu, *J. Am. Chem. Soc.*, 2010, **132**, 17707.

3 S. Soukasene, D. J. Toft, T. J. Moyer, H. Lu, H.-K. Lee, S. M. Standley, V. L. Cryns and S. I. Stupp, *ACS Nano*, 2011, **5**, 9113.

4 V. Jayawarna, M. Ali, T. A. Jowitt, A. E. Miller, A. Saiani, J. E. Gough and R. V. Ulijn, *Adv. Mater.*, 2006, **18**, 611; J. P. Jung, A. K. Nagaraj, E. K. Fox, J. S. Rudra, J. M. Devgun and J. H. Collier, *Biomaterials*, 2009, **30**, 2400.

5 J. S. Rudra, T. Sun, K. C. Bird, M. D. Daniels, J. Z. Gasiorowski, A. S. Chong and J. H. Collier, *ACS Nano*, 2012, **6**, 1557; R. N. Shah, N. A. Shah, M. M. Del Rosario Lim, C. Hsieh, G. Nuber and S. I. Stupp, *Proc. Natl. Acad. Sci. U. S. A.*, 2010; H. Yokoi, T. Kinoshita and S. G. Zhang, *Proc. Natl. Acad. Sci. U. S. A.*, 2005, **102**, 8414; J. B. Matson and S. I. Stupp, *Chem. Commun.*, 2012, **48**, 26.

6 J. S. Rudra, Y. F. Tian, J. P. Jung and J. H. Collier, *Proc. Natl. Acad. Sci. U. S. A.*, 2010, **107**, 622.

7 I. M. Geisler and J. P. Schneider, *Adv. Funct. Mater.*, 2012, **22**, 529.

8 A. R. Hirst, S. Roy, M. Arora, A. K. Das, N. Hodson, P. Murray, S. Marshall, N. Javid, J. Sefcik, J. Boekhoven, J. H. van Esch, S. Santabarbara, N. T. Hunt and R. V. Ulijn, *Nat. Chem.*, 2010, **2**, 1089.

9 Z. J. Qiu, H. T. Yu, J. B. Li, Y. Wang and Y. Zhang, *Chem. Commun.*, 2009, 3342.

10 C. Ren, Z. Song, W. Zheng, X. Chen, L. Wang, D. Kong and Z. Yang, *Chem. Commun.*, 2011, **47**, 1619; C. J. Bowerman and B. L. Nilsson, *J. Am. Chem. Soc.*, 2010, **132**, 9526.

11 X. L. Zhang, X. L. Chu, L. Wang, H. M. Wang, G. L. Liang, J. X. Zhang, J. F. Long and Z. M. Yang, *Angew. Chem., Int. Ed.*, 2012, **51**, 4388.

12 C. Sinthuvanich, A. S. Veiga, K. Gupta, D. Gaspar, R. Blumenthal and J. P. Schneider, *J. Am. Chem. Soc.*, 2012, **134**, 6210.

13 H. Yokoi, T. Kinoshita and S. G. Zhang, *Proc. Natl. Acad. Sci. U. S. A.*, 2005, **102**, 8414.

14 J. S. Rudra, S. Mishra, A. S. Chong, R. A. Mitchell, E. H. Nardin, V. Nussenzweig and J. H. Collier, *Biomaterials*, 2012, **33**, 6476.

15 Y. Wang, L. M. Zhao and B. M. Hantash, *Biomaterials*, 2010, **31**, 5122; A. Mujeeb, A. F. Miller, A. Saiani and J. E. Gough, *Biomaterials*, 2013, **9**, 4609.

16 E. J. Leon, N. Verma, S. Zhang, D. A. Lauffenburger and R. D. Kamm, *J. Biomater. Sci., Polym. Ed.*, 1998, **9**, 297; D. M. Marini, W. Hwang, D. A. Lauffenburger, S. Zhang and R. D. Kamm, *Nano Lett.*, 2002, **2**, 295; W. Hwang, D. M. Marini, R. D. Kamm and S. Zhang, *J. Chem. Phys.*, 2003, **118**, 389.

17 M. R. Caplan, E. M. Schwartzfarb, S. Zhang, R. D. Kamm and D. A. Lauffenburger, *Biomaterials*, 2002, **23**, 219.

18 C. J. Bowerman and B. L. Nilsson, *Biopolymers*, 2012, **98**, 169; C. J. Bowerman, W. Liyanage, A. J. Federation and B. L. Nilsson, *Biomacromolecules*, 2011, **12**, 2735; R. J. Swanekamp, J. T. M. DiMaio, C. J. Bowerman and B. L. Nilsson, *J. Am. Chem. Soc.*, 2012, **134**, 5556; C. J. Bowerman, D. M. Ryan, D. A. Nissan and B. L. Nilsson, *Mol. BioSyst.*, 2009, **5**, 1058.

19 J.-B. Guilbaud, E. Vey, S. Boothroyd, A. M. Smith, R. V. Ulijn, A. Saiani and A. F. Miller, *Langmuir*, 2010, **26**, 11297.

20 X. Du, Z. D. Wu, J. F. Long and L. Wang, *RSC Adv.*, 2013, **3**, 18259.

21 J. Raeburn, G. Pont, L. Chen, Y. Cesbron, R. Levy and D. J. Adams, *Soft Matter*, 2012, **8**, 1168.

22 S. I. Shin, V. H. Freedman, R. Risser and R. Pollack, *Proc. Natl. Acad. Sci. U. S. A.*, 1975, **72**, 4435; M. A. Cifone and I. J. Fidler, *Proc. Natl. Acad. Sci. U. S. A.*, 1980, **77**, 1039; S. Mori, J. T. Chang, E. R. Andrechek, N. Matsumura, T. Baba, G. Yao, J. W. Kim, M. Gatza, S. Murphy and J. R. Nevins, *Oncogene*, 2009, **28**, 2796.

