Anti-degradation of a recombinant complex protein by incoporation in small molecular hydrogels†

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A phenomenon of anti-degradation of a recombinant complex protein (MPP6 complex protein) in a small molecular hydrogel was reported in this study.

Recombinant proteins, even for those being purified by size-exclusion high performance liquid chromatography (SE-HPLC), frequently suffer from degradation by protease, which causes serious problems for their storage, crystal growth, *etc*. The strategy of the addition of protease inhibitors during the purification can greatly improve the stability of the recombinant proteins.[1] However, it is hard to totally inhibit the activity of the whole family of protease by a single inhibitor. What's more, the purified proteins obtained by SE-HPLC usually need to be stored in cold places (*e.g*. in the fridge at low temperatures), which needs a lot of energy and is not convenient during transportation. Therefore, a simple and convenient method that can greatly improve the stability of recombinant proteins at room temperature is highly desirable. Small molecular (SM) hydrogels,[2] formed by the self-assembly of small molecules,[3] might be an ideal candidate because the proteins can be fixed in their three dimensional matrix, thus reducing the possibility of recognition and degradation of proteins by the protease. In this study, we demonstrated in principle that the stability of a recombinant complex protein—membrane-associated guanylate kinases p55 subfamily member 6 (MPP6), residues 1-106AA complexed with Mals3—could be greatly improved by simple incorporation into a SM hydrogel.

It has been proved that SM hydrogels could provide suitable environments for bioactive individuals such as cells,[4] sensors,[5] proteins,[6,7] and drugs.[8] Lots of small molecules based on naturally occuring components have been found to be efficient SM hydrogelators including derivatives of amino acids,[9] sugars,[10] peptides,[6] and drug molecules.[11] In this study, a short peptide-based SM hydrogel of Nap-GFF (***1*** in Fig. 1) developed by our group was chosen due to its versatile synthetic pathway and its excellent gelation ability.[12]

After the successful synthesis of Nap-GFF by solid phase peptide synthesis (SPPS) and purification by reverse phase HPLC, the gelation property of ***1*** was tested by the invert-tube method. The minimum gelation concentration of ***1*** was 0.2 wt% in the phosphate buffered saline (pbs) buffer solution (pH = 7.4), which meant that 1 molecule of ***1*** could gel about 14 950 molecules of H_2O in pbs solutions. This result correlated well with the fact that derivatives of diphenylalanine were efficient gelators for organic solvents and aqueous solutions.[13]

The gel with 1.0 wt% of ***1*** in pbs buffer (gel I) was then used for the encapsulation of the MPP6 complex protein purified by SE-HPLC. Since gel I was a thixotropic hydrogel, it allowed the encapsulation of the protein by a simple vortex. The gel with the MPP6 complex protein (gel II) would re-form after being kept at room temperature (22–25 °C) for about 2 h. The MPP6 complex protein in the pbs buffer and in gel II was then tested by the SDS-PAGE gel electrophoresis. The results shown in Fig. 2 indicated that about 10% of the MPP6 complex protein degraded after 24 h (lane 3) and the protein was totally degraded after 48 h (lane 4) in the pbs buffer solution. However, there was no obvious degradation of the complex protein being observed in gel II at room temperature after 48 h and about 58% of the protein remained in the gel II after 7 days, which indicated that the stability of the MPP6 complex protein was improved by a simple encapsulation in SM gels. To understand the mechanism of stabilization of MPP6 protein in hydrogels, we used a model protein of bovine serum albumin (BSA) to be mixed with MPP6 protein solutions in PBS buffer solutions and in gels. As shown in the supporting information†, the BSA itself was stable in PBS solutions for at least 3 days. However, it would undergo degradation by mixing it with the MPP6 protein solutions in the buffer solutions. Similarly to MPP6 protein, its stability could be

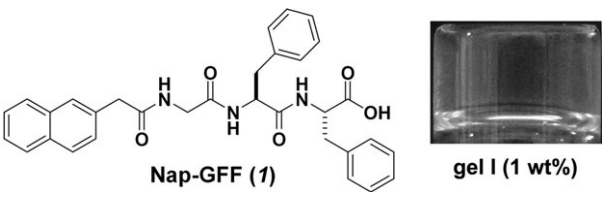

Fig. 1 The chemical structure of Nap-GFF and an optical image of gel I with 1 wt% of ***1*** in PBS buffer (pH = 7.4).

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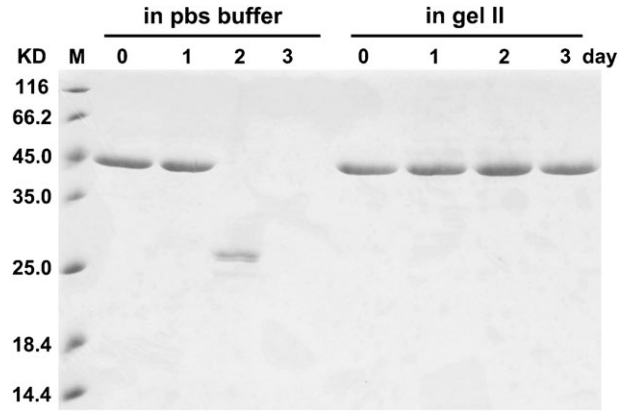

Fig. 2 The scanning image of SDS-PAGE gel electrophoresis of recombinant MPP6 complex protein in the PBS buffer solution (pH = 7.4) and in gel II at day 0–3.

improved by incorporation of it in the SM hydrogels. These results indicated the presence of the digestion enzyme/enzymes in MPP6 protein solutions, which could not be separated from the MPP6 complex protein by SE-HPLC. These results also indicated that such kind of great improvement of the stability of the proteins was due to the fixation of the protein and the digestion enzymes in the fiber networks, which dramatically reduced the diffusion rate of the enzymes and the proteins and decreased the possibility of the proteins being recognized by the digestion enzymes.

In order to test whether the activity of the encapsulated protein had been changed or not, the protein of an enzyme (phosphatase) was incorporated into the gel and its activity was measured at different time points. The results showed that the activity of the phosphatase in the gel after 48 h was almost the same to that of free phosphatase in pbs solution at day 0 (98% of the activity remained),[14] which meant that the self-assembled fiber network had no obvious effects on the activity of the encapsulated enzyme in the short time.

We then went back to characterize both kinds of the hydrogels (gel I and gel II). The mode of dynamic frequency sweep was firstly used to characterize the mechanical property of both gels. As shown in Fig. 3A, the values of G′ of gel I and gel II were higher than those of G″ of gel I and gel II respectively at the low frequency regions, indicating that both gels were solid-like samples. The values of G′ and G″ of both gels showed strong dependencies on the frequency—both values of G′ and G″ increased with enhancement of the frequency and the values of G″ of gel I and gel II were almost the same as those of G′ of both gels respectively at the high frequency regions. Comparing the values of G′ of both gels, it was found that the value of G′ decreased from 120 Pa in gel I to 6 Pa in gel II formed by the heating-cooling cycle after the addition of a small amount of the protein (0.005 wt%). This result suggested that the protein might interact with the small molecules, thus inhibiting the formation of large bundles of the self-assembled structures and weakening the mechanical property of the gels. Since gel I was a thixotropic hydrogel, the mode of dynamic time sweep was used to test its recovery property. As shown in Fig. 3B, both values of G′ and G″ dropped rapidly and only 18% of the original

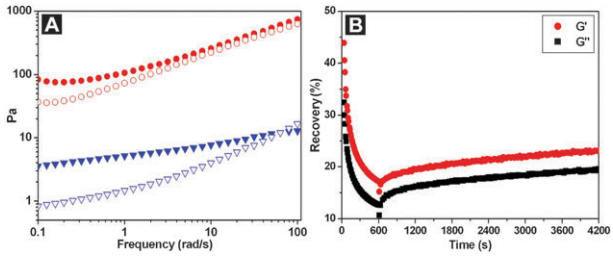

Fig. 3 (A) Dynamic frequency of gel I with 1.0 wt% of ***1*** and gel II with 1.0 wt% of ***1*** and 0.005 wt% of MPP6 complex protein in pbs solutions at the strain of 2% (both gels were formed by the heating-cooling cycle, gel I: circles, gel II: triangles, G′: filled symbols, and G″: open symbols) and (B) the recovery property of gel I with 1.0 wt% of ***1*** (gel I was firstly subjected to a large strain of 50% for 10 min and then its recovery was probed at the strain of 2% and frequency of 2 rad s^{-1}).

value of G′ was observed after 10 min under the external large stress (strain = 50%). After the removal of the external large stress, the speed of recovery of the strength of gel I was quite slow—only 24% of the original value of G′ was achieved after 1 h. These results implied that gels formed by ***1*** were weak and thixotropic hydrogels that possessed a shear thinning and slow recovery property.

Atomic force microscopy (AFM) was used to characterize the nanostructures within the gels (Fig. 4). Small fibrils with a diameter of about 20 nm and ribbons with width of about 60 nm were observed in gels I (Fig. 4A). Both the fibrils and ribbons in gel I were longer than 2 μm and they entangled with each other to form a three dimensional network for gel I. To understand what happened for the gel I before and after vortex, we also obtained the AFM image of gel I after vortex. As shown in the supporting information (Fig. S-7†), gel I after vortex exhibited a relatively higher amount of small fibrils with a size of about 20 nm than gel I before vortex. This result suggested that the ribbons or fibers with larger sizes were formed by the bundles of the small fibrils and the vortex would weaken the interaction between these small fibrils, thus leading to mechanically weaker hydrogels. Compared to gel I before vortex, gel II showed much smaller and uniform fibrils with a diameter of about 15 nm and there were no ribbons being observed (Fig. 4B). These observations correlated well with the result of a much smaller value of G′ of gel II than that of gel I. These results further suggested that the existence of a tiny amount of protein might have strong influences on the microscopic (morphology and molecular arrangement) and macroscopic (mechanical property) properties of the gels.

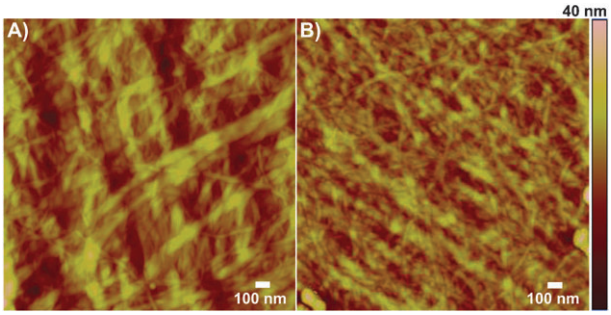

Fig. 4 AFM images of (A) gel I and (B) gel II.

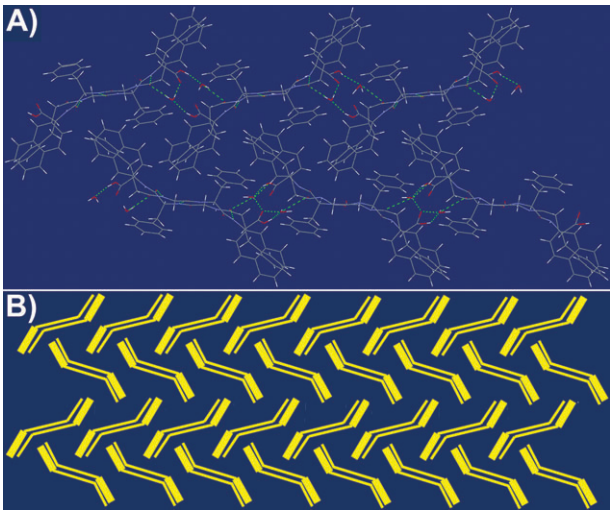

Fig. 5 (A) The crystal structure of Nap-GFF obtained from ethanol and (B) schematic molecular arrangement of ***1*** in self-assembled structures.

The crystal structure of Nap-GFF (Fig. 5) was obtained to understand the molecular packing of ***1*** in the nanostructures. Unlike the Nap-FF, in which the naphthalene group interacted with the phenyl ring of its own phenylalanine (F) and two Nap-FF molecules formed a dimer with a 'X' shape,[15] the naphthalene group on Nap-GFF was far away from the phenyl ring of its own phenylalanine, thus facilitating the formation of the dimer with a '//' shape. This observation helped us to understand the super-gelation ability of the derivatives of Nap-GFF.[16] The '//' shape of the dimer and the four water molecules between two dimers helped the formation of extensive hydrogen bonds between Nap-GFF and both the hydrogen bonds and aromatic interactions helped to efficiently extend the supramolecular chains to form superstructures.

In summary, we demonstrated the first example of the application of SM hydrogels in anti-degradation of complex proteins by a simple encapsulation strategy. Such a strategy would not dramatically decrease the activity of the encapsulated enzyme in the SM hydrogels. Though only 58% of the encapsulated enzyme remained in the gel after storage at room temperature for 7 days, the stability of the encapsulated protein at room temperature might be further improved by using SM gels with higher mechanical property. The results indicated that SM hydrogels not only had potential to be developed into promising biomaterials for long term storage of recombinant proteins at room temperature, but also would be useful for the delivery of proteins to treat different diseases. It was also quite unusual that the addition of a tiny amount of protein caused such dramatic effects on the microscopic and macroscopic properties of the hydrogels, which will be further studied and reported in due course.

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Notes and references

1 P. M. Martensen and J. Justesen, *Biotechniques*, 2001, **30**, 782.
2 M. O. M. Piepenbrock, G. O. Lloyd, N. Clarke and J. W. Steed, *Chem. Rev.*, 2010, **110**, 1960; L. A. Estroff and A. D. Hamilton, *Chem. Rev.*, 2004, **104**, 1201; R. J. Williams, A. M. Smith, R. Collins, N. Hodson, A. K. Das and R. V. Ulijn, *Nat. Nanotechnol.*, 2009, **4**, 19; A. Brizard, M. Stuart, K. van Bommel, A. Friggeri, M. de Jong and J. van Esch, *Angew. Chem., Int. Ed.*, 2008, **47**, 2063; J. P. Jung, J. Z. Gasiorowski and J. H. Collier, *Biopolymers*, 2010, **94**, 49; S. Debnath, A. Shome, D. Das and P. K. Das, *J. Phys. Chem. B*, 2010, **114**, 4407; J. Chen and A. J. McNeil, *J. Am. Chem. Soc.*, 2008, **130**, 16496; K. N. King and A. J. McNeil, *Chem. Commun.*, 2010, **46**, 3511.
3 A. R. Hirst, B. Escuder, J. F. Miravet and D. K. Smith, *Angew. Chem., Int. Ed.*, 2008, **47**, 8002; D. Koda, T. Maruyama, N. Minakuchi, K. Nakashima and M. Goto, *Chem. Commun.*, 2010, **46**, 979; Y. Cao, L. M. Tang, Y. J. Wang, B. Y. Zhang and L. K. Jia, *Chem. Lett.*, 2008, **37**, 554; C. J. Bowerman and B. L. Nilsson, *J. Am. Chem. Soc.*, 2010, **132**, 9526; G. Cheng, V. Castelletto, C. M. Moulton, G. E. Newby and I. W. Hamley, *Langmuir*, 2010, **26**, 4990; D. J. Adams, M. F. Butler, W. J. Frith, M. Kirkland, L. Mullen and P. Sanderson, *Soft Matter*, 2009, **5**, 1856; S. Dutta, A. Shome, S. Debnath and P. K. Das, *Soft Matter*, 2009, **5**, 1607.
4 E. Genove, C. Shen, S. G. Zhang and C. E. Semino, *Biomaterials*, 2005, **26**, 3341; M. Zhou, A. M. Smith, A. K. Das, N. W. Hodson, R. F. Collins, R. V. Ulijn and J. E. Gough, *Biomaterials*, 2009, **30**, 2523.
5 S. Kiyonaka, K. Sada, I. Yoshimura, S. Shinkai, N. Kato and I. Hamachi, *Nat. Mater.*, 2004, **3**, 58; A. Wada, S. Tamaru, M. Ikeda and I. Hamachi, *J. Am. Chem. Soc.*, 2009, **131**, 5321.
6 S. Koutsopoulos, L. D. Unsworth, Y. Nagaia and S. G. Zhang, *Proc. Natl. Acad. Sci. U. S. A.*, 2009, **106**, 4623.
7 S. Kiyonaka, S. Shinkai and H. Hamachi, *Chem.–Eur. J.*, 2003, **9**, 976.
8 S. Sutton, N. L. Campbell, A. I. Cooper, M. Kirkland, W. J. Frith and D. J. Adams, *Langmuir*, 2009, **25**, 10285; Y. J. Seo, S. Bhuniya and B. H. Kim, *Chem. Commun.*, 2007, 1804; A. Shome, S. Debnath and P. K. Das, *Langmuir*, 2008, **24**, 4280.
9 S. Dutta, D. Das, A. Dasgupta and P. K. Das, *Chem.–Eur. J.*, 2010, **16**, 1493; M. Suzuki and K. Hanabusa, *Chem. Soc. Rev.*, 2009, **38**, 967.
10 S. Kiyonaka, K. Sugiyasu, S. Shinkai and I. Hamachi, *J. Am. Chem. Soc.*, 2002, **124**, 10954.
11 S. Bhuniya, Y. J. Seo and B. H. Kim, *Tetrahedron Lett.*, 2006, **47**, 7153; Y. Gao, Y. Kuang, Z. F. Guo, Z. H. Guo, I. J. Krauss and B. Xu, *J. Am. Chem. Soc.*, 2009, **131**, 13576; J. A. Saez, B. Escuder and J. F. Miravet, *Tetrahedron*, 2010, **66**, 2614.
12 Y. H. Hu, H. M. Wang, J. Y. Wang, S. B. Wang, W. Liao, Y. G. Yang, Y. J. Zhang, D. L. Kong and Z. M. Yang, *Org. Biomol. Chem.*, 2010, **8**, 3267.
13 A. Mahler, M. Reches, M. Rechter, S. Cohen and E. Gazit, *Adv. Mater.*, 2006, **18**, 1365; X. H. Yan, P. L. Zhu and J. B. Li, *Chem. Soc. Rev.*, 2010, **39**, 1877; X. H. Yan, Y. Cui, Q. He, K. W. Wang and J. B. Li, *Chem. Mater.*, 2008, **20**, 1522.
14 See the supporting information.
15 Z. M. Yang, H. W. Gu, J. Du, J. H. Gao, B. Zhang, X. X. Zhang and B. Xu, *Tetrahedron*, 2007, **63**, 7349.
16 H. M. Wang, C. H. Ren, Z. J. Song, L. Wang, X. M. Chen and Z. M. Yang, *Nanotechnology*, 2010, **21**, 225606.