Nanostructures of β-sheet peptides: steps towards bioactive functional materials

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The design and construction of synthetic self-assembled nanostructures is, in a large part, inspired from elaborate nanostructures in biological systems. If we look at it from another angle, the self-assembled nanostructures are excellent scaffolds for exploring and modulating biological phenomena when they are suitably functionalized with bioactive molecules. Of the many types of molecular building blocks for self-assembly, peptide-based building blocks have the advantage in that their constituent amino acids are biocompatible and the sequence space of peptide chains is vast. This contribution highlights an emerging approach to the biomaterial application of artificially designed and functional β-sheet peptide self-assemblies.

The construction of exquisite supramolecular nanostructures through molecular self-assembly of synthetic molecules has been investigated intensively during the past decade, which is in particular due to the potential to develop novel nano-scale materials.[1] Given the fact that a basic principle of supramolecular chemistry is the iterative and regular array of monomeric building blocks, the self-assembled nanostructures are excellent platforms for displaying multiple functionalities.[2] The numbers of monomeric units in the supramolecular nanostructures (aggregation number), varying from two to hundreds of thousands of units, are dependent on the structure of the monomeric building blocks and the solution environment. In a biological system, myriads of molecular recognition events take place in a multivalent fashion.[3] Multivalent interactions provide a significant increase in binding affinity that is not achievable with monovalent interactions. Synthetic multivalent molecules are useful in inhibiting or modulating biological interactions.

Peptide-based self-assembling systems are increasingly investigated for the construction of supramolecular structures, which have potential applications as biocompatible multivalent scaffolds. The peptides usually assemble through α-helical, β-sheet, and hydrophobic interactions.[4–7] Examples of peptide-based self-assembly systems include micelles from peptide amphiphiles,[8] coiled-coils from α-helical peptide bundles,[9] nanotubes from cyclic peptides,[10] nanotubes and nanocages from dipeptides,[11] vesicles from diblock peptides of polylysine or polyarginine and polyleucine,[12] thermoresponsive elastin-like aggregates,[13] closed-micelles from peptide–PEG block copolymers,[14] and nanofibers from β-sheet peptides.[15–18] The peptide nanostructures have mostly been used for applications in drug delivery, gene delivery, and antimicrobial agent development.

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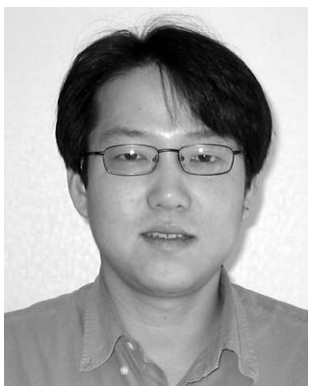

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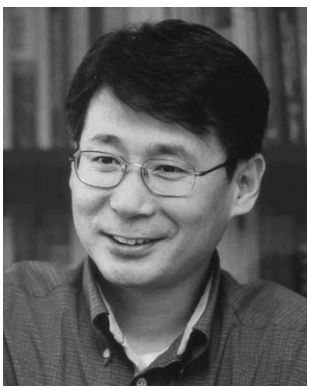

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Myongsoo Lee received his BS degree in chemistry from Chungnam National University, Korea and PhD degree in Macromolecular Science from Case Western Reserve University, Cleveland, in 1992. In the same year, he became a postdoctoral fellow at University of Illinois, Urbana-Champaign. In 1994, he joined the Faculty of Chemistry at Yonsei University, Korea, where he is presently Professor of Chemistry. Presently, he is a director of the Center for Supramolecular Nano-Assembly and a member of the editorial board of Chemistry – An Asian Journal*. His current research interests include synthetic self-organizing molecules, controlled supramolecular architectures, and organic nanostructures with biological functions.*

Among the peptide nanostructures, the β-sheet peptide nanostructures are likely to be especially suitable for applications where fibrous structures are advantageous, such as crosslinking cells and *in vivo* delivery experiments. It has been demonstrated that the nanoparticles with filamentous shape persist longer than those with spherical shape under fluid flow conditions present *in vivo*.[19]

β-Sheet peptide nanostructures

Artificially designed β-sheet peptides have gained growing attention for their potential to be used as biomaterials.[15–18] The polypeptide chains are nearly fully extended in β-sheet structures in which regular hydrogen bonds form between the peptide backbone amide protons and carbonyl oxygen groups of adjacent chains (Fig. 1a). The β-sheet structure, along with the α-helix, is the one of the main secondary structural elements in proteins.[20] The adjacent β-strands can lie in either a parallel or an antiparallel fashion. In both parallel and antiparallel β-sheets, the β-strands have conformations pointing alternate amino acid side chains to opposite sides of the sheet. The peptide chains in antiparallel β-sheets (a repeat period of 7.0 Å/residue pair) are slightly more extended than those in parallel β-sheets (a repeat period of 6.5 Å/residue pair). The repeat period refers to the average distance along the chain axis of each dipeptide unit. Contributions from electrostatic and hydrophobic forces between amino acid side chains on the same face of the sheet often help to stabilize the sheets.

Nanofiber of β-sheets are organized in such a way that each β-strand runs perpendicular to the fibril axis. The design principle for most of the artificial β-sheet peptides is the alternating placement of charged (or polar) and hydrophobic amino acids. This type of arrangement promotes the proper β-sheet hydrogen bonding arrangement between amide hydrogen and carbonyl oxygen. When one face of the one-dimensional β-sheet (β-tape) consists predominantly of hydrophobic side chains, the removal of the hydrophobic side chains from contact with water drives two β-tapes to associate into a bilayered β-ribbon structure. This type of bilayered β-ribbon structure is reminiscent of a β-sandwich structure, which is largely stabilized by hydrophobic interactions and is one of the most common topologies of β-sheet structures found in proteins (Fig. 1b).[20]

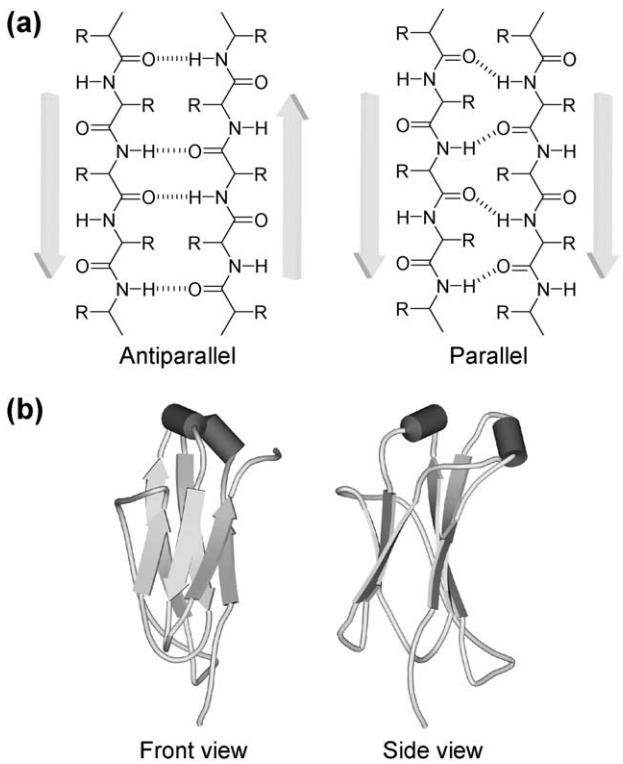

Fig. 1 (a) Two forms of the β-sheet structure, the antiparallel and the parallel β-sheet. (b) An example of a β-sandwich structure. The diagram was created using coordinates (PDB entry 1MCS) for immunoglobulin λ light chain dimer.

It has been demonstrated that many peptides having a propensity to β-sheet nanofiber formation often laterally interact to form higher order aggregates.[16] Coupling of hydrophilic macromolecules on the N- or C-terminus of β-sheet peptides significantly inhibits the higher order aggregates' formation. For example, it has been found that coupling of polyethylene glycol (PEG) on the β-sheet peptide can suppress the lateral aggregation of β-sheet nanostructures and enhance their solubility in aqueous solution (Fig. 2a).[15c,16]

In addition to constructing simple nanofibers of β-sheet peptide, several strategies have been developed to make stimuli responsive β-sheet peptide nanostructures.[17] Many carefully designed β-sheet peptide nanostructures have been shown to be responsive to pH, salts, ionic strength, and enzymatic stimuli to make the transition from sol to gel, from nematic to isotropic phase, and from random coil to β-sheet. These findings have offered a way to generate discrete and smart nanostructures of β-sheet peptides, which should be important steps towards making functional materials.

β-Sheet peptide nanostrutures with biological functions

If the β-sheet peptides are conjugated with bioactive functions, it would be possible to construct bio-functional multivalent nanofibers. In one attempt, a 16-amino acid residue β-sheet peptide (β16) was co-assembled with a biotinylated β16 (Fig. 2b).[18a] Then the streptavidin modified with colloidal gold was added to the mature nanofibers. Transmission electron microscopy (TEM) investigation revealed that the gold particles were attached to the nanofibers at regular intervals due to the molecular co-assembly. This study implies that a variety of functional molecules can be immobilized on peptide nanofibers in controlled distance and amount. In direct conjugation strategies, protein molecules such as green fluorescent protein (GFP) and cytochrome were successfully attached in their active forms to β-sheet nanofibers.[18b,c] In a recent report, the β-sheet fiber forming a SH_3 domain

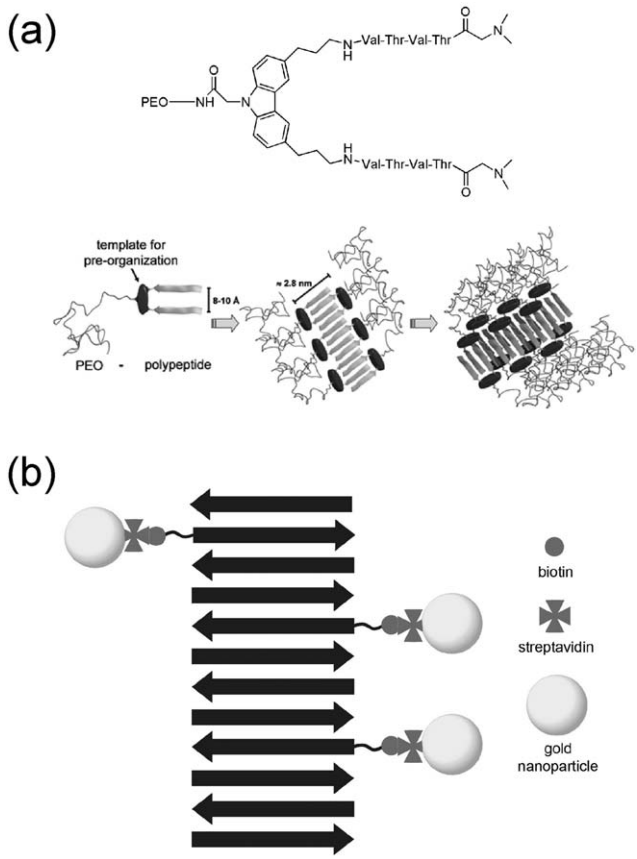

Fig. 2 (a) Artificially designed PEG-coated β-sheet nanostructure. Reproduced with permission in part from ref. 15*c*, © 2005 Royal Society of Chemistry. (b) Introduction of model functional groups (gold nanoparticles) into the cofibrils containing biotinylated β-sheet peptides.

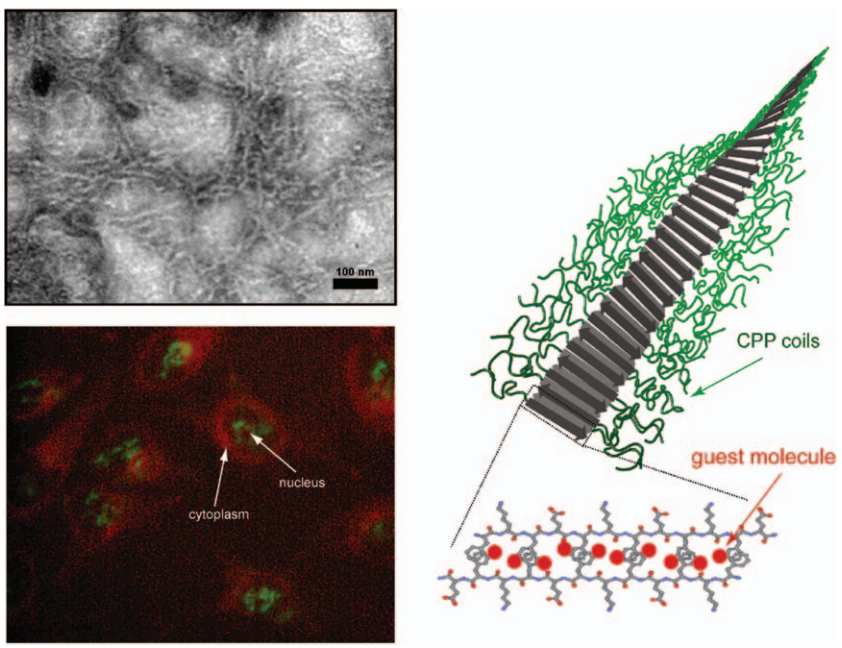

Fig. 3 CPP-coated β-ribbon and intracellular delivery of encapsulated guest molecules. In a confocal laser scanning microscope image of the cells, TβP and encapsulated guest molecules are shown in green and red, respectively. Reproduced in part from ref. 18*d*, © 2007 Wiley-VCH Verlag GmbH & Co. KGaA. Used with permission.

protein was fused with cytochrome (Cyt), a porphyrin binding protein that catalyzes redox reaction in the cell.[18c] TEM, X-ray diffraction (XRD), and circular dichroism (CD) analyses showed that the fusion protein forms β-sheet fibers coated densely with cytochromes. The UV-vis spectra of the $(SH_3)_2$Cyt fibrils bound to iron(ii) and iron(iii) protoporphyrin IX were identical to those of the wild type Cyt and other spectroscopic analyses further confirmed that activity of Cyt was not impaired by the fibril formation. The results demonstrate that this fibril provides a novel nanostructured material for the study of charge transfer in supramolecular systems.

We have recently reported that the self-assembling block peptide (TβP) of a cell penetrating peptide (CPP) Tat and a β-sheet assembly peptide (FKFEFK-FEFKFE) was designed and synthesized for intracellular delivery applications (Fig. 3).[18d] It was found that TβP formed nanoribbons in which β-sheet interaction was the main driving force for the self-assembly. The TβP nanoribbon was able to encapsulate hydrophobic guest molecules such as pyrene and nile red in the hydrophobic interface between two β-tapes, showing the possibility of use in drug delivery applications similarly to conventional amphiphilic block copolymer micelles.[21] It turned out that the cell penetration efficiency of the TβP nanoribbon was much higher than that of unimolecular Tat CPP, suggesting that the multiple coating of CPPs is advantageous in increasing cellular uptake efficiency.[22]

In another example, carbohydrate-functionalized β-sheet nanostructures were developed to agglutinate bacterial cells.[18e,f] Agglutination of microbial cells such as bacteria and viruses might be developed as a way to inactivate pathogens. The building block consists of a carbohydrate mannose, a oligo(ethylene glycol) and a β-sheet assembly peptide (Fig. 4). GP1 and GP2 building blocks were found to form long and short β-ribbons, respectively. To investigate interactions between the mannose-coated β-ribbons and the bacterial cells, we chose an *E. coli* strain containing mannose-binding adhesin FimH in its type 1 pili (ORN178) as a model pathogen.[23] Upon addition of the mannose-coated long β-ribbon from GP1 to *E. coli* ORN178 suspension, the bacteria lost their motility and agglutinated,

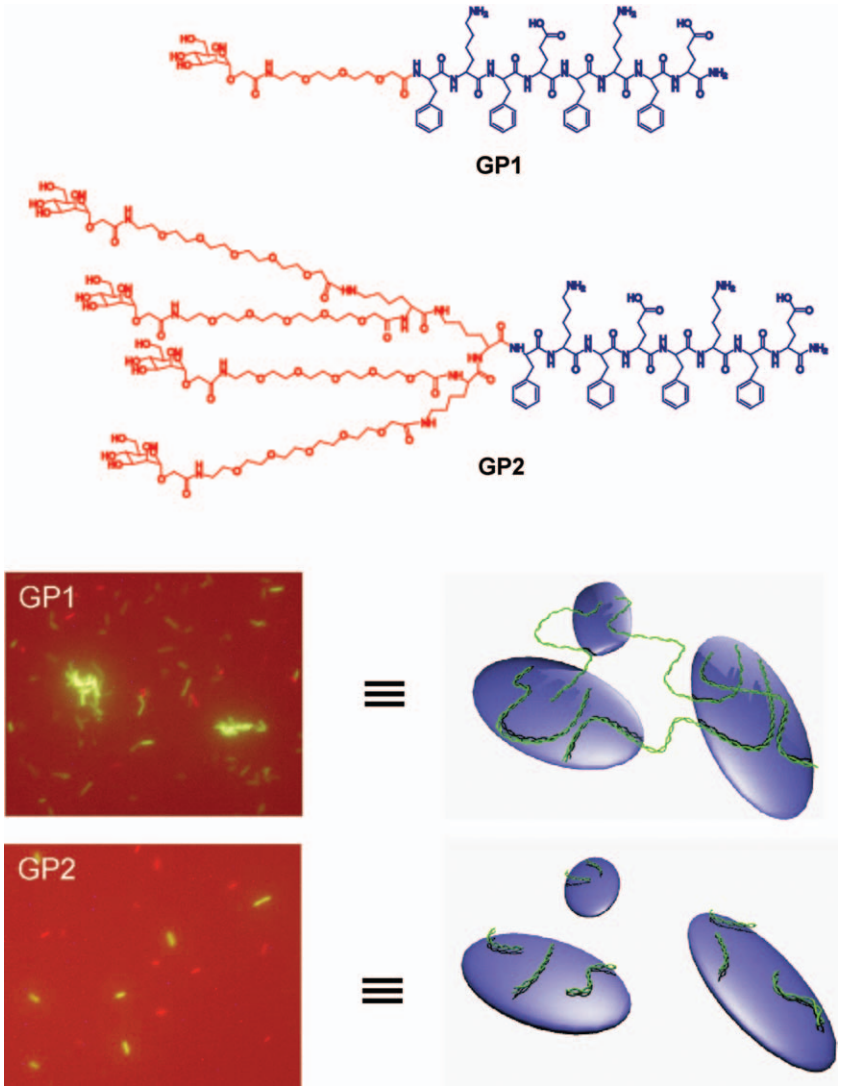

Fig. 4 Overlaid fluorescence microscopy images of fluorescent bacteria. GP1 nanoribbons induced bacterial agglutination. ORN178-GFP *E. coli* (specific to mannose), green; ORN208-RFP *E. coli* (non-specific to mannose), red. Reproduced in part from ref. 18*e*, © 2007 American Chemical Society. Used with permission.

whereas short β-ribbons from GP2 only inhibited bacterial motility. A similar observation has recently been reported that carbohydrate-coated long carbon nanotubes could aggregate anthrax spores, whereas carbohydrate-coated spherical nanoparticles could not.[24] Overall, these observations stress one significant point regarding the interactions at the supramolecular level: the size and morphology of nanostructures are critically important even if their chemical properties are similar.[25]

Conclusion

In principle, any type of functional molecule can be attached to β-sheet peptides for generating functional materials. However, in reality, one should take many parameters into account for the nanostructures to self-assemble well and to be functional. For example, it has been reported that coating of large-sized proteins might weaken the formation of β-sheet secondary structures.[26] Therefore, a balance should be maintained between the segments of opposing forces, *i.e.*, a β-sheet assembly peptide segment and a hydrophilic functional segment. Proper design of supramolecular building blocks and judicious utilization in appropriate biotechnological applications will certainly be necessary.

Compared to amphiphilic α-helical peptides which usually form spherical vesicular structures,[12] β-sheet peptides mostly self-assemble into fibrous structures, which often lead to the formation of higher order aggregates. Certainly, more systematic studies are necessary to overcome the difficulties associated with forming well-defined β-sheet assemblies by higher order aggregate formation. In addition, finding a general strategy to control the size/length of the β-sheet fiber by adjusting the number of strand associations would be an important issue for future research.[15a,f,g,16c,17c,18e,f] These issues are important not only for developing functional β-sheet peptide materials, but also for understanding and inhibiting protein misfolding such as in amyloid fibrogenesis.

Other types of functional molecules, *e.g.*, DNA, RNA, and small bioactive molecules, can also be targets for display in β-sheet nanostructures. The majority of synthetic tasks for β-sheet peptides can be done using automated peptide synthesizers. Therefore, compared to other organic self-assembly systems, the β-sheet peptide self-assembly system might be easier to be approached by one who does not have strong expertise in synthetic chemistry and high-throughput studies would be possible. Given the interdisciplinary nature of the subject, it is necessary to put knowledge in material science, chemistry, biology, and biomedical science together for the field to advance further.

Acknowledgements

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References

1 (*a*) J.-M. Lehn, *Proc. Natl. Acad. Sci. U. S. A.*, 2002, **99**, 4763–4768; (*b*) L. Brunsveld, B. J. B. Folmer, E. W. Meijer and R. P. Sijbesma, *Chem. Rev.*, 2001, **101**, 4071–4097; (*c*) T. Shimizu, M. Masuda and H. Minamikawa, *Chem. Rev.*, 2005, **105**, 1401–1443; (*d*) M. Lee, B.-k. Cho and W.-C. Zin, *Chem. Rev.*, 2001, **101**, 3869–3892; (*e*) D. E. Discher and A. Eisenberg, *Science*, 2002, **297**, 967–973; (*f*) J. A. A. W. Elemans, A. E. Rowan and R. J. M. Nolte, *J. Mater. Chem.*, 2003, **13**, 2661–2670; (*g*) K. Kinbara and T. Aida, *Chem. Rev.*, 2005, **105**, 1377–1400; (*h*) A. Mueller and D. F. O'Brien, *Chem. Rev.*, 2002, **102**, 727–757; (*i*) D. Chen and

M. Jiang, *Acc. Chem. Res.*, 2005, **38**, 494–502.
2 (*a*) Y.-b. Lim and M. Lee, *Org. Biomol. Chem.*, 2007, **5**, 401–405; (*b*) K. Larsen, M. B. Thygesen, F. Guillaumie, W. G. T. Willats and K. J. Jensen, *Carbohydr. Res.*, 2006, **341**, 1209–1243; (*c*) X. Chen, U. C. Tam, J. L. Czlapinski, G. S. Lee, D. Rabuka, A. Zettl and C. R. Bertozzi, *J. Am. Chem. Soc.*, 2006, **128**, 6292–6293; (*d*) J. E. Kingery-Wood, K. W. Williams, G. B. Sigal and G. M. Whitesides, *J. Am. Chem. Soc.*, 1992, **114**, 7303–7305; (*e*) G. Thoma, A. G. Katopodis, N. Voelcker, R. O. Duthaler and M. B. Streiff, *Angew. Chem., Int. Ed.*, 2002, **41**, 3195–3198; (*f*) B. S. Kim, W. Y. Wang, J. H. Ryu, Y. S. Yoo and M. Lee, *Chem. Commun.*, 2005, 2035–2037; (*g*) B. S. Kim, D. J. Hong, J. Bae and M. Lee, *J. Am. Chem. Soc.*, 2005, **127**, 16333–16337.
3 (*a*) S.-K. Choi, *Synthetic multivalent molecules*, John Wiley & Sons, Inc., New Jersey, 2004; (*b*) M. Mammen, S. K. Choi and G. M. Whitesides, *Angew. Chem., Int. Ed.*, 1998, **37**, 2755–2794; (*c*) J. S. Kim and C. O. Pabo, *Proc. Natl. Acad. Sci. U. S. A.*, 1998, **95**, 2812–2817; (*d*) C. R. Bertozzi and L. L. Kiessling, *Science*, 2001, **291**, 2357–2364; (*e*) J. J. Lundquist and E. J. Toone, *Chem. Rev.*, 2002, **102**, 555–578; (*f*) Y. C. Lee and R. T. Lee, *Acc. Chem. Res.*, 1995, **28**, 321–327.
4 S. X. Ye, J. W. Strzalka, B. M. Discher, D. Noy, S. Y. Zheng, P. L. Dutton and J. K. Blasie, *Langmuir*, 2004, **20**, 5897–5904.
5 S. G. Zhang, *Nat. Biotechnol.*, 2003, **21**, 1171–1178.
6 S. Fernandez-Lopez, H. S. Kim, E. C. Choi, M. Delgado, J. R. Granja, A. Khasanov, K. Kraehenbuehl, G. Long, D. A. Weinberger, K. M. Wilcoxen and M. R. Ghadiri, *Nature*, 2001, **412**, 452–455.
7 J. D. Hartgerink, E. Beniash and S. I. Stupp, *Science*, 2001, **294**, 1684–1688.
8 (*a*) G. A. Silva, C. Czeisler, K. L. Niece, E. Beniash, D. A. Harrington, J. A. Kessler and S. I. Stupp, *Science*, 2004, **303**, 1352–1355; (*b*) K. Rajangam, H. A. Behanna, M. J. Hui, X. Han, J. F. Hulvat, J. W. Lomasney and S. I. Stupp, *Nano Lett.*, 2006, **6**, 2086–2090.
9 (*a*) M. M. Stevens, N. T. Flynn, C. Wang, D. A. Tirrell and R. Langer, *Adv. Mater.*, 2004, **16**, 915–918; (*b*) M. J. Pandya, G. M. Spooner, M. Sunde, J. R. Thorpe, A. Rodger and D. N. Woolfson, *Biochemistry*, 2000, **39**, 8728–8734; (*c*) E. B. Hadley and S. H. Gellman, *J. Am. Chem. Soc.*, 2006, **128**, 16444–16445; (*d*) L. J. Leman, D. A. Weinberger, Z.-Z. Huang, K. M. Wilcoxen and M. R. Ghadiri, *J. Am. Chem. Soc.*, 2007, **129**, 2959–2966.
10 (*a*) M. R. Ghadiri, J. R. Granja, R. A. Milligan, D. E. Mcree and N. Hazanovich, *Nature*, 1993, **366**, 324–327; (*b*) J. H. van Maarseveen, W. S. Horne and M. R. Ghadiri, *Org. Lett.*, 2005, **7**, 4503–4506.
11 (*a*) M. Reches and E. Gazit, *Science*, 2003, **300**, 625–627; (*b*) S. Ghosh, M. Reches, E. Gazit and S. Verma, *Angew. Chem., Int. Ed.*, 2007, **46**, 2002–2004.
12 (*a*) E. P. Holowka, V. Z. Sun, D. T. Kamei and T. J. Deming, *Nat. Mater.*, 2007, **6**, 52–57; (*b*) E. P. Holowka, D. J. Pochan and T. J. Deming, *J. Am. Chem. Soc.*, 2005, **127**, 12423–12428.
13 (*a*) D. W. Urry, C.-H. Luan, T. M. Parker, D. C. Gowda, K. U. Prasad, M. C. Reid and A. Safavy, *J. Am. Chem. Soc.*, 1991, **113**, 4346–4348; (*b*) D. E. Meyer and A. Chilkoti, *Biomacromolecules*, 2004, **5**, 846–851; (*c*) A. Valiaev, D. W. Lim, T. G. Oas, A. Chilkoti and S. Zauscher, *J. Am. Chem. Soc.*, 2007, **129**, 6491–6497.
14 Y. Lee, S. Fukushima, Y. Bae, S. Hiki, T. Ishii and K. Kataoka, *J. Am. Chem. Soc.*, 2007, **129**, 5362–5363.
15 (*a*) G. T. Dolphin, P. Dumy and J. Garcia, *Angew. Chem., Int. Ed.*, 2006, **45**, 2699–2702; (*b*) C. W. G. Fishwick, A. J. Beevers, L. M. Carrick, C. D. Whitehouse, A. Aggeli and N. Boden, *Nano Lett.*, 2003, **3**, 1475–1479; (*c*) D. Eckhardt, M. Groenewolt, E. Krause and H. G. Börner, *Chem. Commun.*, 2005, 2814–2816; (*d*) W. H. Binder and O. W. Smrzka, *Angew. Chem., Int. Ed.*, 2006, **45**, 7324–7328; (*e*) H. Shao, J. W. Lockman and J. R. Parquette, *J. Am. Chem. Soc.*, 2007, **129**, 1884–1885; (*f*) K. Janek, J. Behlke, J. Zipper, H. Fabian, Y. Georgalis, M. Beyermann, M. Bienert and E. Krause, *Biochemistry*, 1999, **38**, 8246–8252; (*g*) H. Dong, S. E. Paramonov, L. Aulisa, E. L. Bakota and J. D. Hartgerink, *J. Am. Chem. Soc.*, 2007, **129**, 12468–12472.
16 (*a*) T. S. Burkoth, T. L. S. Benzinger, D. N. M. Jones, K. Hallenga, S. C. Meredith and D. G. Lynn, *J. Am. Chem. Soc.*, 1998, **120**, 7655–7656; (*b*) J. H. Collier and P. B. Messersmith, *Adv. Mater.*, 2004, **16**, 907–910; (*c*) J. M. Smeenk, M. B. J. Otten, J. Thies, D. A. Tirrell, H. G. Stunnenberg and J. C. M. van Hest, *Angew. Chem., Int. Ed.*, 2005, **44**, 1968–1971.
17 (*a*) R. J. Mart, R. D. Osborne, M. M. Stevens and R. V. Ulijn, *Soft Matter*, 2006, **2**, 822–835; (*b*) J. Hentschel, E. Krause and H. G. Börner, *J. Am. Chem. Soc.*, 2006, **128**, 7722–7723; (*c*) R. Mimna, M.-S. Camus, A. Schmid, G. Tuchscherer, H. A. Lashuel and M. Mutter, *Angew. Chem., Int. Ed.*, 2007, **46**, 2681–2684.
18 (*a*) H. Kodama, S. Matsumura, T. Yamashita and H. Mihara, *Chem. Commun.*, 2004, 2876–2877; (*b*) N. Sondheimer and S. Lindquist, *Mol. Cell*, 2000, **5**, 163–172; (*c*) A. J. Baldwin, R. Bader, J. Christodoulou, C. E. MacPhee, C. M. Dobson and P. D. Barker, *J. Am. Chem. Soc.*, 2006, **128**, 2162–2163; (*d*) Y.-b. Lim, E. Lee and M. Lee, *Angew. Chem., Int. Ed.*, 2007, **46**, 3475–3478; (*e*) Y.-b. Lim, S. Park, E. Lee, H. Jeong, J.-H. Ryu, M. S. Lee and M. Lee, *Biomacromolecules*, 2007, **8**, 1404–1408; (*f*) Y.-b. Lim, S. Park, E. Lee, J.-H. Ryu, Y.-R. Yoon, T.-H. Kim and M. Lee, *Chem.–Asian J.*, 2007, **2**, 1363–1369.
19 Y. Geng, P. Dalhaimer, S. Cai, R. Tsai, M. Tewari, T. Minko and D. E. Discher, *Nat. Nanotechnol.*, 2007, **2**, 249–255.
20 (*a*) C. L. Nesloney and J. W. Kelly, *Bioorg. Med. Chem.*, 1996, **4**, 739; (*b*) H. J. Dyson, J. R. Sayre, G. Merutka, H. C. Shin, R. A. Lerner and P. E. Wright, *J. Mol. Biol.*, 1992, **226**, 819.
21 (*a*) H. Otsuka, Y. Nagasaki and K. Kataoka, *Adv. Drug Delivery Rev.*, 2003, **55**, 403–419; (*b*) R. Haag and F. Kratz, *Angew. Chem., Int. Ed.*, 2006, **45**, 1198–1215; (*c*) R. Savić, L. Luo, A. Eisenberg and D. Maysinger, *Science*, 2003, **300**, 615–618.
22 M. Sung, G. M. K. Poon and J. Gariépy, *Biochim. Biophys. Acta*, 2006, **1758**, 355–363.
23 S. L. Harris, P. A. Spears, E. A. Havell, T. S. Hamrick, J. R. Horton and P. E. Orndorff, *J. Bacteriol.*, 2001, **183**, 4099–4102.
24 H. Wang, L. Gu, Y. Lin, F. Lu, M. J. Meziani, P. G. Luo, W. Wang, L. Cao and Y.-P. Sun, *J. Am. Chem. Soc.*, 2006, **128**, 13364–13365.
25 J.-H. Ryu, E. Lee, Y.-b. Lim and M. Lee, *J. Am. Chem. Soc.*, 2007, **129**, 4808–4814.
26 U. Baxa, V. Speransky, A. C. Steven and R. B. Wickner, *Proc. Natl. Acad. Sci. U. S. A.*, 2002, **99**, 5253.