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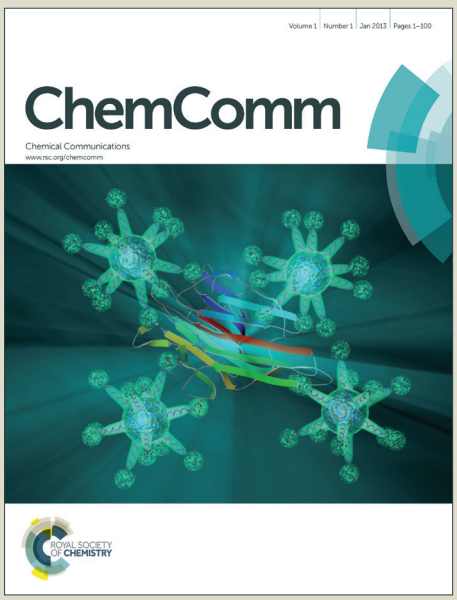

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Reversible Thermo-sensitivity Induced from Varied Hydrogen Bonding between Side Residues of Rationally Designed Polypeptides

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Rationally designed polypeptides with similar molecular structure but varying patterns of hydrogen bonding between side groups have been synthesized and demonstrated to possess distinct solubility and thermal behavior. Further balancing the ratio of both isopropylamine and ethylenediamine side groups endows the random copolymer with reversible thermo-sensitivity.

Hydrogen bonding has become one of the most widely used approaches in supramolecular chemistry to form hierarchical structures which are difficult to obtain *via* conventional ways[1-3]. In particular, polypeptides organize themselves in an ordered way to proteins mainly *via* intra- or inter-molecular interactions including hydrogen bonding in the biological systems. Originated from the repeating amide bonds of the polypeptide backbone, α-helix and β-sheet are considered as two basic secondary structures for proteins[4]. It has been demonstrated that except for the covalent bonding, the secondary or tertiary structure of proteins enables bioactivities function properly[5-7]. Therefore, it is of great importance to predict, control and tune the hierarchical structure of polypeptides, ultimately leading to the desired property. To mimic and extent the application of natural biopolymers, several attempts have been made to achieve well-defined 3 dimensional structures from synthetic polypeptides[8-10]. Among these, rationally molecular design has received special attention due to its versatile and feasible nature. For example, foldamers that combined β- or γ-amino acids with natural α-amino acids can fold into a conformationally ordered state with the main goal of moving from structure to function[11, 12]. Peptoid is known as pseudo-peptide with *N*-substituted in the backbone of peptide. The corresponding polymer exhibits high solubility and excellent thermal properties in the absence of hydrogen bonds[13-15]. However, the synthetic procedure for foldamers and polypeptoids was usually tedious or the starting monomer was limited to few amino acids[16].

Thanks to the rapid progress in *N*-carboxyanhydride (NCA) polymerization, synthetic polypeptides with high yield and l[illegible] quantity have been realized from various natural amino acids[17]. Combined with advanced modification techniques, tailor-mad[illegible] polypeptide materials with manipulated structures and properti[illegible] were developed. These materials are highly demanded in the biomedical fields such as drug/gene delivery and tissue engineering due to the excellent biodegradability and biocompatibility of the building α-amino acid units[18, 19]. Furthermore, those α-amino ac[illegible] with reactive side group could be easily incorporated with different functionalities to construct stimuli responsive polypeptides ("smart" polypeptides)[20, 21]. Basically there are two routes to decorate a synthetic polypeptide chain regardless of copolymerization wi[illegible] other units[22]. In the first route, natural amino acids reacted with the target groups followed by the formation of NCAs, which were further polymerized. This route has been extensively investigated in the past few decades because protection and deprotection of the reactive side groups were avoided and a complete functionalizatio[illegible] was guaranteed. Deming *et al*. firstly reported the synthesis of PEGylated NCA of L-lysine, L-cysteine and L-serine, which w[illegible] polymerized for water-soluble properties by changing the length of OEG side chains[23, 24]. It was illustrated by Dong *et al*. that PEO initiated ring opening polymerization (ROP) of *S*-(*o*-nitrobenzyl)-L-cysteine NCA resulted in the micelles for phototriggered drug-release.[25] However, post-polymerization modification (the second route) could also be selected for those reactive side groups easily deprotected and clickable. Heise *et al*. reported that amphiphilic block copolymer of poly(γ-benzyl-L-glutamate)-*b*-poly(galactosylated propargyl glycine) was prepared by Huisgen [3+2] cylcoaddition with azide-functional galactose[26]. It was further investigated that their self-assembly behavior could be well controlled depending on the block composition[27]. Hammond *et al*. have demonstrated a dual temperature and pH responsive system through 1,3-cycloaddition reaction between poly(γ-propargyl-L-glutamate) and alkyne, where the solubility and secondary structure could be tuned by the ratio of substitution. As stated by Schlaad *et al*., well-defined copolymers of glycosylated and non-glycosylated polyglutamtate by thiol-ene/yne photo-chemistry could undergo pH induced conformation transition.[29] In some critical cases, both routes have synchronously been applied for the complex primary structure and multi-functionality. Zhang *et al*. have shown that azido and allyl dual-functionalized diblock copolypeptide were prepared from ROP of γ-allyl-L-glutamic acid NCA and *g*-3-chloropropyl-[illegible]

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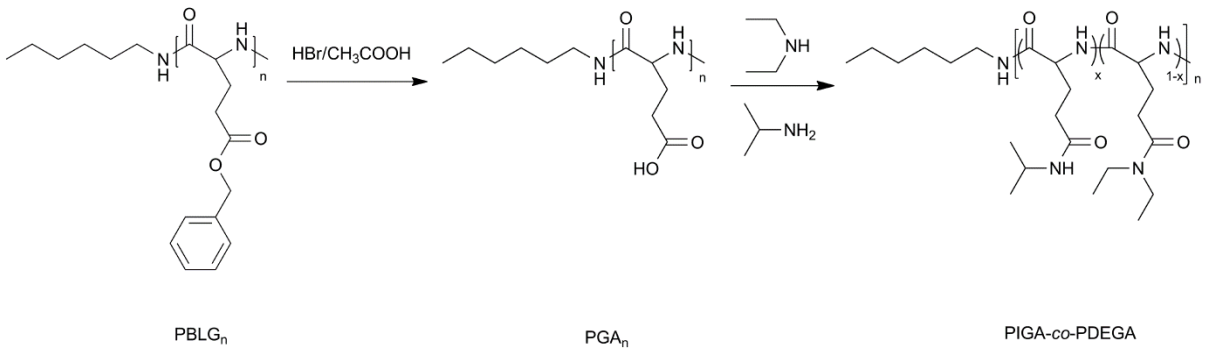
glutamic acid NCA, followed by a nucleophilic substitution with NaN_3. The modified copolypeptide allowed for grafting propargylmannose and 3-mercaptopropionic acid by copper-mediated alkyne-azide [2+3] cycloaddition and radical thiol-ene addition reactions, respectively. The resulted copolypeptide with diverse structures by side-chain conjugation had a good control over bioactivity, solubility and self-assembly properties[30].



Scheme 1 Synthetic routes of homopolymers PIGA, PDEGA and random copolymers PIGA-*co*-PDEGA

Although many synthetic polypeptides were successfully prepared, how to direct and predict their secondary structure with the aim to tune the material property remains an enormous challenge. The pattern of hydrogen bonds within the polypeptide backbone is likely to be the dominant factor that bridges the gap between molecular design and material property. A few insights have been given in the correlation of some important properties like thermosensivity with the ordering structures of polypeptide backbone. It has been reported by Chen *et al*. that subtle variation in alkyl side group displayed significant effect on the secondary structure and gelation behavior of thermosensitive polypeptide block copolymer based on PEG and poly(L-glutamate)[31]. Li *et al*. introduced different repeating OEG units onto cysteine[32] and L-glutamate[8], whose corresponding homopolypeptides varied in hierarchical structures as well as temperature-dependent properties. Charged poly(γ-(4-(1-hexanol-6-aminomethyl))benzyl-L-glutamate) was designed by Cheng *et al*. to obtain remarkable helical stability and water solubility[33]. However, the abovementioned researchers mainly focused on the conformational change of the backbone by introduction of side ester/ether functionality, which could not act as a hydrogen bond donor. Providing that the side group/chain had been modified with an amide bond, the patterns of hydrogen bonding both on the backbone and the side group would have coordinately directed the structure ordering, which might endow the material with unique properties. In this study, we present a model that fine tuning between hydrogen bond donor and acceptor on the side group of a synthetic polypeptide could discriminate its solubility in water. Further balancing the ratio of both side groups enables the material sensitive to temperature variation.

Amide bond was chosen as the side group in our synthetic polypeptide due to its diversity in the hydrogen bonding formation[34-37]. However, ROP of NCAs from glutamine and asparagine consisting of amide substitute has rarely been reported[38, 39]. The difficulties in synthesis and polymerization of their NCAs might have hindered the development of such amide functionalized polypeptides. Therefore, we carried out aminolysis of the side carboxyl group on poly(L-glutamate) for a feasible synthetic procedure and mutable modification. Both isopropylamine and ethylenediamine were respectively reacted with pending carboxyl groups on the poly(L-glutamate) (PGA) (Scheme 1, x stands for 0 or 1), which was derived from poly(γ-benzyl-L-glutamate) (PBLG) by deprotection as reported elsewhere[40, 41]. The grafted primary amine resulted in the amide bond, which represents not only a donor but also an acceptor of hydrogen bonding on the side chain of poly(*N*-isopropyl-L-glutamine) (PIGA). While poly(*N*-diethyl-L-glutamine) (PDEGA) with secondary amine grafting reserved highly similar molecular structure with PIGA, the formed *N*-alkyl amide bond could only serve as a hydrogen bond acceptor. It was agreed with our hypothesis that the model polypeptide with more possibility of patterns of hydrogen bonding induced from side chain was built. The ^{1}H NMR spectra and MALDI-TOF MS (Fig S1, S4 and S5, ESI†) confirmed the exact molecular structure of both PIGA and PDEGA.

Table 1 Some parameters of the synthesized homopolymers and copolymers

Entry	Polymers	IGA (mol%)[a]	T_g (°C)[b]	Water Solubility
P1	$PIGA_{20}$	100	96.39	--
P2	$PIGA_{15}$-*co*-$PDEGA_5$	75	82.02	--
P3	$PIGA_{13}$-*co*-$PDEGA_7$	65	80.28	--
P4	$PIGA_{11}$-*co*-$PDEGA_9$	55	78.55	--
P5	$PIGA_9$-*co*-$PDEGA_{11}$	45	73.46	--
P6	$PIGA_7$-*co*-$PDEGA_{13}$	35	71.57	+
P7	$PIGA_5$-*co*-$PDEGA_{15}$	20	70.27	+
P8	$PIGA_3$-*co*-$PDEGA_{17}$	15	68.66	+
P9	$PDEGA_{20}$	0	67.64	+

[a] Determined by ^{1}H NMR. [b] Determined by DSC (Fig S3, ESI†).
+ Well solubility. -- Poor solubility.

Interestingly, PDEGA displayed high water solubility while PIGA remained insoluble at a relatively dilute state (0.5 mg/ml). This phenomenon promoted us to further investigate the secondary structure of both homopolymers, which were designed at a comparable level with the same DP of 20. (Table 1, Entry P1 and P9). The number average molecular weight calculated from GPC, NMR and MALDI-TOF MS (Table S2, Fig S1, S4, and S5, ESI†) was consistent with the theoretical value, demonstrating that the polymerization and post-polymerization modification were controllable. Except for the extreme difference in water solubility, the thermal property of both homopolymers appeared significantly distinct. The glass transition temperature (T_g) of PIGA (96.4 °C) was much higher than that of PDEGA (67.6 °C). These preliminary results indicated that the inter-/intra-molecular interaction in PIGA might be stronger than that in PDEGA.

Hydrogen bonding was considered as the most dominant factor of molecular interaction in our system. Therefore, FTIR and CD spectroscopy were applied to study the conformation of both synthetic polypeptides. As shown in Fig. 1, the strong amide I of PIGA appeared at 1653 cm^{-1}, indicating that most of PIGA chains adopted α-helix conformation, which was attributed to intramolecular hydrogen bonds. Meanwhile, the absorption band at 1627 cm^{-1} was observed for PDEGA, in which the backbone folded mainly into β-sheet by intermolecular hydrogen bonding. It was hinted from FTIR results that the molecular interaction between side chains might dominate the different backbone conformation. We further looked into CD spectra of both PIGA and PDEGA in water as shown in Fig. 2. It was found that PIGA exhibited a positive cotton band at 193 nm and two negative ones at 207 nm and 223 nm, which were three characteristic cotton bands of α-helix. In contrast, a positive cotton band at 192 nm and a negative cotton band at 209 nm indicated β-sheet structure of $PDEGA_{20}$. It was highly consistent that both FTIR and CD spectra revealed diverse backbone conformation for PIGA and PDEGA. Considering their high similarity in primary structure, the hydrogen bonding between side chains may lead to the different ordering of the backbone.

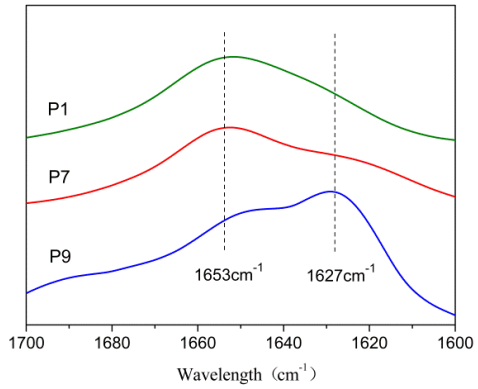


Fig. 1 FTIR spectra of entry **P1**, **P7** and **P9** in the solid state.

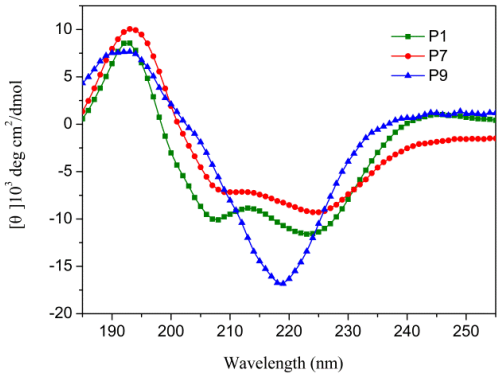


Fig. 2 Circular dichroism spectra of entry **P1**, **P7** and **P9** (0.5 mg/mL) in the aqueous solution

Relating the backbone conformation with the properties observed, we proposed a possible model for the patterns of hydrogen bonding in PIGA and PDEGA. It is known that every α-helix cycle contains 3.6 amino acids along one molecular chain with side residues towards outside. For the helical PIGA, it is very likely to form hydrogen bonding between side residues because of their regular and close-range packing, as illustrated in Fig. 3. However, β-sheet consists of β-strand connected laterally along the backbone, forming a generally twisted, pleated sheet with the side group alternatively distributing on the surface. It is apparent that α-helix ordering of backbone in PIGA may drive the side amide bond to form more hydrogen bonds than β-sheet, which led to a less intensive packing of side residues. Therefore, PIGA preferred α-helix conformation instead of β-sheet. Because of the high density of hydrogen bonds within both the backbone and side residues, PIGA with a high T_g is difficult to dissolve in water for the lack of free H bond donor/acceptor. For PDEGA, hydrogen bonding could not be formed between side *N*-alkyl amide groups. Therefore, the backbone adopted a β-sheet conformation for a loose packing of side chains in terms of steric hindrance (Fig. 3). Moreover, the N in *N*-alkyl amide bonds could conjugate with water easily due to its higher electronegative character as an excellent H-bond acceptor. It is explained that PDEGA with less hydrogen bonding between side groups appeared a lower T_g and better solubility in water.

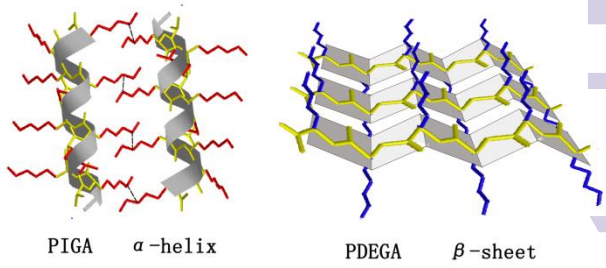


Fig. 3 Schematic illustration of hierarchical structure of polypeptide PIGA (α-helix) and PDEGA (β-sheet).

Stimulated by the extremely different water-solubility of PDEGA and PIGA abovementioned, thermoresponsive polypeptide would be expected by carefully balancing their ratio[42]. Therefore, a series of random copolymers of PIGA-*co*-PDEGA were designed and synthesized with different ethyldiamine/isopropylamine compositions (Scheme 1, x varying between 0 and 1). Both ^{1}H NMR spectra and GPC results (Fig S1 and Table S2, ESI†) indicated that the desired molecular structure, composition and DP were achieved. The solubility testing revealed that the copolymers became more water-soluble with increasing ratio of PDEGA segments. There were only three copolymers, *i.e.*, P6, P7 and P8, retaining acceptable water solubility. Furthermore, T_g of all the copolymers, listed in Table 1, decreased almost linearly from 96.4 °C 69.6 °C with PDEGA component increasing from 0 to 100%. These results coincided with our proposed model that more hydrogen bonds between side residues in PIGA led to poor solubility and higher T_g.

Temperature-dependent ultraviolet spectra were performed to investigate thermoresponsive behavior of the water soluble polymers P9, P8, P7 and P6 (Fig S6, ESI†). It was suggested that only P7 exhibited a definite lower critical solution temperature (LCST), which was characterized as a clear transition from transparent to turbid with temperature increasing. Moreover, a reversible LCST behavior was observed for P7 when recovering the sample to room temperature as illustrated in Fig. 4a. To understand the underlying mechanism regarding the driving force of LCST behavior, temperature-dependent ^{1}H NMR of P7 ($PIGA_5$-*co*-$PDEGA_{15}$) was conducted to explore the local chemical environmental variation of characteristic proton versus temperature changes (Fig. 4b). At room temperature, the peaks for protons of methylene (δ 3.4) and methyne

(δ 4.3) were easily identified for PIGA and PDEGA respectively. With the temperature increasing especially above its LCST, it was observed that the methenyl peak disappeared while the methylene peak significantly shifted and decreased, suggesting that dehydration of both PIGA and PDEGA side chains occurred with temperature enhancement. Therefore, a "poly(*N*-isopropylacrylamide) (PNIPAM)" like copolypeptide with inherent biodegradability and biocompatibility was designed and synthesized by tuning the hydrogen bonding ability with water.

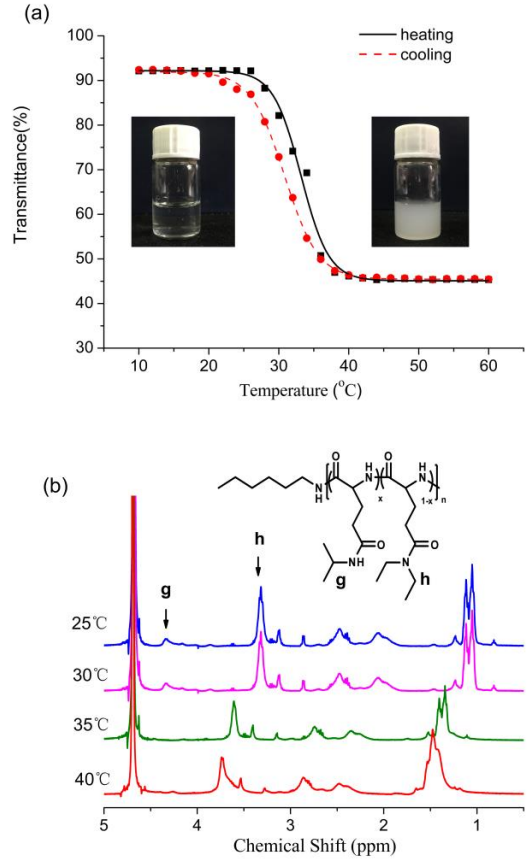


Fig. 4. (a) Plots of transmittance as a function of temperature for aqueous solution of **P7** (2 mg/mL). Solid line: heating; dashed line: cooling. (b) Temperature-dependent ^{1}H NMR for **P7** in aqueous solution (30 mg/mL).

In conclusion, homopolymers and copolymers of PIGA and PDEGA have been successfully synthesized *via* post-polymerization modification approach with well-defined DP and composition. Hydrogen bonds between side residues in PIGA are considered to dominate the backbone conformation, further leading to its poor water-solubility and higher T_g. While *N*-alkyl amide bond represents hydrogen acceptor on the side chain, PDEGA exhibited better water solubility and lower T_g without hydrogen bonds between side groups but strong water bonding ability. Further balancing the ratio of PIGA and PDEGA resulted in a thermal reversible copolypeptide, which mimics PNIPAM with its own biodegradability and biocompatibility. Our preliminary results of such synthetic polypeptides provided more understanding on the patterns of hydrogen bonding, which would promote elegant designs for more hierarchical structures and novel biomedical materials.

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Notes and references

1. L. M. Pitet, A. H. M. van Loon, E. J. Kramer, C. J. Hawker and E. W. Meijer, *ACS Macro Lett.*, 2013, **2**, 1006-1010.
2. Y. Chen, X.-H. Pang and C.-M. Dong, *Adv. Funct. Mater.*, 2010, **20**, 579-586.
3. M. Guo, L. M. Pitet, H. M. Wyss, M. Vos, P. Y. Dankers and E. Meijer, *J. Am. Chem. Soc.*, 2014, **136**, 6969-6977.
4. C. Cai, J. Lin, Z. Zhuang and W. Zhu, in *Controlled Polymerization and Polymeric Structures*, Springer, 2013, pp. 159-199.
5. F. Monnaie, W. Ceunen, J. De Winter, P. Gerbaux, V. Cocchi, E. Salatelli and G. Koeckelberghs, *Macromolecules*, 2015, **48**, 90-98.
6. M. Gkikas, J. S. Haataja, J. Seitsonen, J. Ruokolainen, O. Ikkala, H. Iatrou and N. Houbenov, *Biomacromolecules*, 2014, **15**, 3923-3930.
7. J. R. Kramer and T. J. Deming, *J. Am. Chem. Soc.*, 2012, **134**, 4112-4115.
8. C. Chen, Z. Wang and Z. Li, *Biomacromolecules*, 2011, **12**, 2859-2863.
9. A. F. Mehl, S. P. Feer and J. S. Cusimano, *Biomacromolecules*, 2012, **13**, 1244-1249.
10. J. Shen, C. Chen, W. Fu, L. Shi and Z. Li, *Langmuir*, 2013, **29**, 6271-6278.
11. L. Berlicki, L. Pilsl, E. Weber, I. M. Mandity, C. Cabrele, T. A. Martinek, F. Fulop and O. Reiser, *Angew. Chem.-Int. Edit.*, 2012, **51**, 2208-2212.
12. S. Chatterjee, P. G. Vasudev, S. Raghothama, C. Ramakrishnan, N. Shamala and P. Balaram, *J. Am. Chem. Soc.*, 2009, **131**, 5956-5965.
13. R. J. Simon, R. S. Kania, R. N. Zuckermann, V. D. Huebner, D. A. Jewell, S. Banville, S. Ng, L. Wang, S. Rosenberg and C. K. Marlowe, *Proc. Natl. Acad. Sci. U. S. A.*, 1992, **89**, 9367-9371.
14. D. Zhang, S. H. Lahasky, L. Guo, C.-U. Lee and M. Lavan, *Macromolecules*, 2012, **45**, 5833-5841.
15. S. H. Lahasky, L. Lu, W. A. Huberty, J. Cao, L. Guo, J. C. Garno and D. Zhang, *Polym. Chem.*, 2014, **5**, 1418-1426.
16. C. Secker, S. M. Brosnan, R. Luxenhofer and H. Schlaad, *Macromolecular bioscience*, 2015, DOI: 10.1002/mabi.201500023.
17. H. R. Kricheldorf, *Angew. Chem.-Int. Edit.*, 2006, **45**, 5752-5784.
18. C. Deng, J. Wu, R. Cheng, F. Meng, H.-A. Klok and Z. Zhong, *Prog. Polym. Sci.*, 2014, **39**, 330-364.
19. C. Bonduelle and S. b. Lecommandoux, *Biomacromolecules*, 2013, **14**, 2973-2983.
20. G. J. M. Habraken, K. H. R. M. Wilsens, C. E. Koning and A. Heise, *Polym. Chem.*, 2011, **2**, 1322.
21. J. Huang and A. Heise, *Chem. Soc. Rev.*, 2013, **42**, 7373-7390.
22. S. Zhang and Z. Li, *J. Polym. Sci. Pt. B-Polym. Phys.*, 2013, **51**, 546-555.
23. J. Hwang and T. J. Deming, *Biomacromolecules*, 2001, **2**, 17-21.
24. M. Yu, A. P. Nowak, T. J. Deming and D. J. Pochan, *J. Am. Chem. Soc.*, 1999, **121**, 12210-12211.
25. G. Liu and C. M. Dong, *Biomacromolecules*, 2012, **13**, 1573-1583.
26. J. Huang, G. Habraken, F. Audouin and A. Heise, *Macromolecules*, 2010, **43**, 6050-6057.
27. J. Huang, C. Bonduelle, J. Thévenot, S. b. Lecommandoux and A. Heise, *J. Am. Chem. Soc.*, 2011, **134**, 119-122.
28. C. M. Chopko, E. L. Lowden, A. C. Engler, L. G. Griffith and P. T. Hammond, *ACS Macro Lett.*, 2012, **1**, 727-731.
29. K.-S. Krannig and H. Schlaad, *J. Am. Chem. Soc.*, 2012, **134**, 18542-18545.
30. H. Tang and D. Zhang, *Polym. Chem.*, 2011, **2**, 1542-1551.
31. Y. Cheng, C. He, C. Xiao, J. Ding, X. Zhuang, Y. Huang and X. Chen, *Biomacromolecules*, 2012, **13**, 2053-2059.
32. X. Fu, Y. Shen, W. Fu and Z. Li, *Macromolecules*, 2013, **46**, 3753-3760.
33. Y. Zhang, H. Lu, Y. Lin and J. Cheng, *Macromolecules*, 2011, **44**, 6641-6644.
34. Y. You, Y. Chen, C. Hua and C. M. Dong, *J. Polym. Sci. Pol. Chem.*, 2010, **48**, 709-718.
35. T. Kimura, S. Takahashi, S. Akiyama, T. Uzawa, K. Ishimori and I. Morishima, *J. Am. Chem. Soc.*, 2002, **124**, 11596-11597.
36. D. W. Lowik, E. H. Leunissen, M. van den Heuvel, M. B. Hansen and J. C. van Hest, *Chem. Soc. Rev.*, 2010, **39**, 3394-3412.
37. V. R. Pattabiraman and J. W. Bode, *Nature*, 2011, **480**, 471-479.
38. D. Ben-Ishai and E. Katchalski, *J. Am. Chem. Soc.*, 1952, **74**, 3688-3689.
39. R. Hirschmann, H. Schwam, R. Strachan, E. Schoenewaldt, H. Barkemeyer, S. Miller, J. B. Conn, V. Garsky, D. F. Veber and R. G. Denkewalter, *J. Am. Chem. Soc.*, 1971, **93**, 2746-2754.
40. E. Blout and M. Idelson, *J. Am. Chem. Soc.*, 1956, **78**, 497-498.
41. Y. Wang and Y. C. Chang, *Macromolecules*, 2003, **36**, 6503-6510.
42. L. Yu, W. Fu and Z. Li, *Soft Matter*, 2015, **11**, 545-550.