Synthesis and bioactivity of poly(HPMA)–lysozyme conjugates: the use of novel thiazolidine-2-thione coupling chemistry

Lei Tao, Jingquan Liu, Jiangtao Xu and Thomas P. Davis*

Received 7th April 2009, Accepted 8th June 2009
First published as an Advance Article on the web 6th July 2009
DOI: 10.1039/b907061c

A novel thiazolidine-2-thione functionalized chain transfer agent (CTA) was synthesized and used as a reversible addition–fragmentation chain transfer (RAFT) polymerization agent to prepare well-defined poly-*N*-(2-hydroxypropyl) methacrylamide (PHPMA). The polymer chains had pre-designed molecular weights, narrow polydispersities and were chain-end functionalized. On incubation with protein (lysozyme) under different pH conditions, PHPMA was conjugated to the protein surface *via* covalent amide bonding. The bioactivity of the lysozyme–PHPMA conjugates was assessed using *Micrococcus lysodeikticus* (Ml) cells as substrates. The number of polymer chains attached to the protein could be controlled by both the pH of the conjugation reaction and the molecular weights of the polymers, thereby influencing significantly the bioactivity of the protein–polymer conjugates.

Introducton

The use of proteins/peptides as therapeutics for clinical applications has developed rapidly with the discovery of novel proteins/peptides and a better understanding of their mechanism *in vivo*.[1–3] However, there are several limitations to the use of protein-based medicines, for example, proteins *in vivo* are vulnerable to renal excretion or digestion by the proteolytic system. Following pioneering attempts to covalently link poly(ethylene glycol) (PEG) to proteins (PEGylation),[4] it has been found that PEG, a synthetic polymer, can protect proteins from recognition by the immune system and hence increase the protein circulation time. PEGylated proteins have been clinically proven to be less toxic and have prolonged plasma circulation time, both are critical factors in reducing the frequency of administration, consequently, immunological side effects are reduced.[5,6] Although the biological activity of PEGylated protein *in vitro* is often dramatically decreased, the improved bioavailability and reduced immunogenicity *in vivo* enhances the efficiency and safety of the therapy.[7]

Living radical polymerization (LRP) yields polymers with predetermined molecular weights and narrow molecular weight distributions, both are desirable properties of well-defined polymer–protein conjugates. Haddleton's and Maynard's groups have reported the use of atom transfer radical polymerization (ATRP)[8–11] to conjugate polymers to proteins. Later, they also developed 'grafting-from' methods to make protein–polymer conjugates by growing polymer chains from pre-prepared protein-initiators.[12–14] The CAMD group extended this synthetic approach by preparing a protein-chain transfer agent (CTA) yielding protein–polymer conjugates by reversible addition–fragmentation chain transfer (RAFT) polymerization.[15,16] After these initial reports, many other polymers have been synthesized by LRP for use in protein conjugation reactions.[17–25]

Centre for Advanced Macromolecular Design (CAMD), School of Chemical Sciences and Engineering, The University of New South Wales, Sydney, NSW 2052, Australia. E-mail: t.davis@unsw.edu.au; Fax: +61-2-9385 6250; Tel: +61-2-9385 4371

N-(2-Hydroxypropyl) methacrylamide (HPMA) has attracted a lot of interest since it was synthesized in the 1960's, as polyHPMA is a neutral, nontoxic, biocompatible and nonimmunogenic polymer.[26,27] Research on PolyHPMA and its copolymers has been focused on the delivery of anti-cancer drugs, tumor-specific antisense oligonucleotides and site-specific delivery to the gastrointestinal (GI) tract.[28–33] However, polyHPMA has not been used for protein modification (analogous with PEGylation) as polyHPMA is normally synthesized by free radical polymerization producing broad molecular weight distributions. Well-defined polyHPMA was not synthesized until 2005, when the McCormick group first reported on the preparation of well-defined polyHPMA using RAFT polymerization.[34] Herein, we report the synthesis of polyHPMA terminated with a thiazolidine-2-thione functional group, that can be used to couple with the amine residues on a protein surface *via* covalent amide bonding to form protein–polyHPMA conjugates. The synthesis of thiazolidine-2-thione terminated polyHPMAs and subsequent conjugation to lysozyme are shown in Scheme 1.

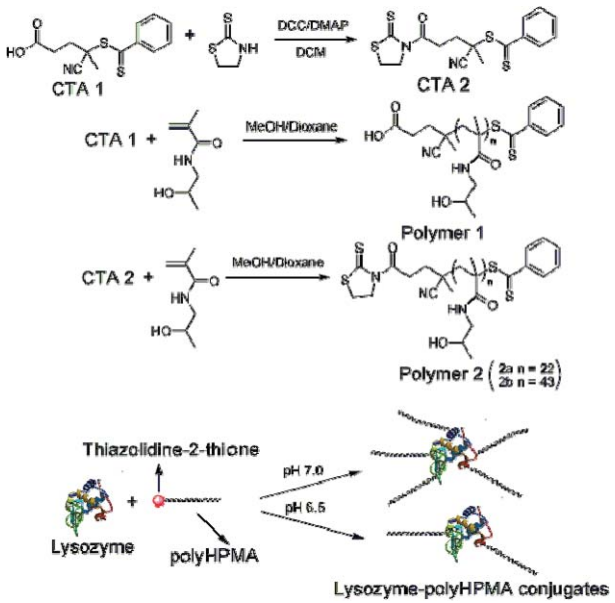
Experimental

1 Materials

2-Mercaptothiazoline (98%, Aldrich), *N*,*N*′-dicyclohexylcarbodiimide (DCC, 99%, Sigma), 4-(dimethylamino) pyridine (DMAP, 99%, Aldrich), *N*-(2-hydroxy propyl) methacrylamide (HPMA, PolyScience), *Micrococcus lysodeikticus* (Ml cell, Sigma) and lysozyme (from chicken egg white, Sigma) were used as purchased. 4-Cyano-4-(phenylcarbonothioylthio) pentanoic acid (CTA 1) was synthesized as described previously.[35] 2,2′-Azobis(isobutyronitrile) (AIBN, 98%, Sigma-Aldrich) was recrystallized twice from acetone. Dichloromethane (DCM, 99%, Ajax) was stored over calcium hydride and distilled before using.

2 Measurement

Gel permeation chromatography (GPC) analyses of polymers were performed using *N*,*N*-dimethylacetamide (DMAc) (0.03% w/v



Scheme 1 Synthesis of thiazolidine-2-thione terminated polyHPMA and subsequent conjugation to lysozyme.

LiBr, 0.05% BHT stabilizer) as the eluent, at 50 °C (flow rate: 0.85 mL min^{-1}) using a Shimadzu modular system comprising a DGU-12A solvent degasser, an LC-10AT pump, a CTO-10A column oven, and an RID-10A refractive index detector. The system was equipped with a Polymer Laboratories 5.0 mm bead-size guard column (50 × 7.8 mm^2) followed by four 300 × 7.8 mm^2 linear PL columns (10^5, 10^4, 10^3, and 500). Calibration was performed with narrow polydisperse polystyrene standards ranging from 500 to 10^6 g mol^{-1}.

Aqueous GPC characterization of protein and protein–polymer conjugates was carried out using a Shimadzu modular system comprising a DGU-12A solvent degasser, a LC-10AT pump, a CTO-10A column oven, and a RID-10A refractive index detector and an SPD-10A Shimadzu UV-Vis spectrometer (flow rate: 1.0 mL min^{-1}). The column was equipped with a Polymer Laboratories 5.0 mm bead-size guard column (50 × 7.8 mm^2) followed by three PL aquagel-OH columns (50, 40, 30, 8 μm). Calibration was performed with PEO standards ranging from 500 to 500 000 g mol^{-1}. The UV-vis absorption spectra were recorded on a Cary 300 Scan spectrophotometer (Varian). ^{1}H NMR spectra were obtained using a Bruker AC300F (300 MHz) spectrometer or a Bruker DPX300 (300 MHz) spectrometer. Multiplicities were reported as singlet (s), broad singlet (bs), doublet (d), triplet (t), and multiplet (m). Mass spectra were obtained on a Finnigan LCQ Deca ion trap mass spectrometer (Thermo Finnigan, San Jose, CA) equipped with an atmospheric pressure ionization source operating in the nebulizer-assisted electrospray mode. The instrument was calibrated in the m/z range 195–1822 Da using a standard containing caffeine, Met-Arg-Phe-Ala acetate salt (MRFA), and a mixture of fluorinated phosphazenes (Ultramark 1621) (all from Aldrich).

3. Methods

3.1 Synthesis of 2-cyano-5-oxo-5-(2-thioxothiazolidin-3-yl) pental-2-yl benzodithioate (CTA 2). CTA 1 (1.68 g, 6.0 mmol), 2-mercaptothiazoline (0.60 g, 5.0 mmol) and DMAP (50 mg, 0.41 mmol) were dissolved in dry DCM; DCC (1.44 g, 7.0 mmol) was added under nitrogen atmosphere. The system was stirred at 25 °C for 6 hours, the solid was filtered and the solvent was removed under vacuum. The crude product was purified by column chromatography on silica gel (ethyl acetate–hexanes = 1 : 3) to yield the product as a red oil. (1.63 g, 85.7%). ^{1}H NMR (300.18 MHz, d_6-DMSO)/ppm: 7.92–7.90 (m, 2H, CHC=S), 7.72–7.67 (m, 1H, CHC*H*CH), 7.54–7.49 (m, 2H, C*H*CHC=S), 4.51 (t, $J = 7.7$ *Hz*, 2H, NCH_2), 3.54–3.41 (m, 2H, $CH_2C{=}O$), 3.38 (t, J = 7.7 Hz, 2H, SCH_2), 2.65–2.47 (m, 2H, CCH_2CH_2), 1.93 (s, 3H, CH_3); ^{13}C NMR (75.49 MHz, d_6-DMSO)/ppm: 223.77, 202.51, 172.06, 144.02, 133.63, 129.04, 126.38, 118.65, 56.30, 45.91, 33.83, 32.38, 28.52, 23.44. IR (cm^{-1}): 1692, 1442, 1364, 1277, 1221, 1153, 1045, 1000, 867. ESI-MS: M + K^+ expected (observed): 418.98 (419.00). Anal. Calcd. for $C_{16}H_{16}N_2OS_4$: C, 50.50; H, 4.24; N, 7.36; S, 33.70; Found: C, 50.50; H, 4.26; N, 7.34; S, 33.73.

3.2 Preparation of polyHPMA. A typical polymerization procedure was as follows: HPMA (0.50 g, 3.48 mmol), CTA 2 (13.3 mg, 0.035 mmol), and AIBN (1.7 mg, 0.01 mmol) were dissolved in methanol–dioxane (5 : 1) (3.0 mL). Aliquots were transferred to five different vials, which were then sealed with rubber septa. Each vial was deoxygenated by purging nitrogen for 30 min prior to placement in a preheated water bath at 65 °C. The vials were removed at 5, 9, 15, 18, and 24 h. Immediate cooling with ice and exposure to air quenched the polymerizations. The monomer conversion for each polymerization sample was determined by ^{1}H NMR directly. After removal of volatile solvents from the polymerization mixtures under vacuum, the residues were redissolved in DMAc for GPC analysis. The final polymers were collected after precipitation (twice) from methanol to diethyl ether and then dried under vacuum. CTA 1 was employed to prepare carboxylic acid terminated PolyHPMA using the same procedure.

3.3 Preparation of protein–polymer conjugates. A typical synthesis procedure for the protein–polymer conjugates (at pH 6.5) was as follows: freshly prepared lysozyme solution (0.5 mL, 3.42×10^{-5} mmol, 1.0 mg mL^{-1} in PBS buffer, pH 6.5) was added to three different polymer samples: polymer **1** (5.0 mg, 0.0014 mmol), polymer **2a** (4.9 mg, 0.0014 mmol) and polymer **2b** (9.2 mg, 0.0014 mmol) The solutions were kept at 25 °C with gentle shaking for 20 h. The solutions were used for bioactivity testing and GPC analyses directly. After removing the salt in the solution by centrifugation filtration (MWCO: 5000), the concentrated solution was used for SDS-PAGE analysis.

3.4 Bioactivity test of the protein–polymer conjugates. The bioactivity of the conjugates was tested using Ml cells as the substrate.[36] Ml cells (4.3 mg) were suspended in PBS buffer solution ((33.0 mL, pH 7.0). An aliquot of the suspension (3.0 mL) was transferred to a cuvette. The initial absorbance at wavelength 450 nm was defined as the baseline. Subsequently lysozyme solution (5 μL of 1.0 mg mL^{-1} in PBS buffer, pH 6.5) was added and the absorbance was measured every 15 seconds for 3 minutes. The lysozyme concentration was calculated from the equation A (unit mL^{-1}) $= -K/(0.001V)$, where A is defined as the relative lysozyme concentration, K is the slope of graph and V is the volume (mL) of lysozyme solution. The data were used as a control in the calculation of the retention of bioactivity of

protein–polymer conjugates, as shown in Fig. 4. The protein–polymer conjugates were tested in a similar fashion with conjugation solution (100 μL) added to the Ml cell suspension, followed by absorbance analyses (450 nm) as detailed above.

Results and discussion

Polymer synthesis

Carboxylic acid (CTA1, polymer **1**) and thiazolidine-2-thione (CTA2, polymer **2**) terminated polyHPMA were prepared *via* RAFT polymerizations. CTA2 exerted control over the polymerization of HPMA, as expected, with linear pseudo-first order kinetic plots (Fig. 1a) and a linear growth of molecular weight with monomer conversions.

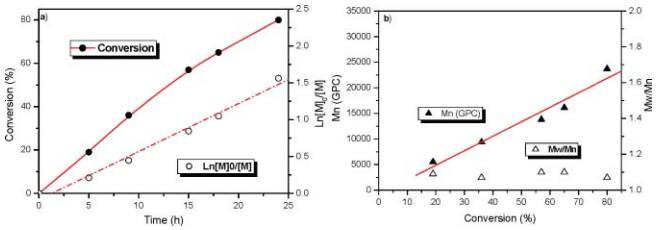


Fig. 1 Polymerization of HPMA using CTA 2 in methanol–dioxane (5 : 1) at 65 °C ($[M]_0 : [CTA\ 2] : [AIBN] = 100 : 1 : 0.30$). (a) Monomer conversions and the kinetic curve *versus* polymerization time; (b) molecular weight (M_w) and PDI of the polymer *versus* monomer conversion.

All the polymers had narrow molecular weight distributions (PDI ≤1.10) (Fig. 1b), indicative of a well-controlled RAFT process. The ^{1}H NMR spectra of polymer **1** (Fig. 2a) and polymer **2** (Fig. 2b) show signals at 7.81 ppm from the two protons of the phenyl group (both CTAs, signal a in Fig. 2). A signal corresponding to the methylene group (CH_2S) on the thiazoline-2-thione moiety is seen at 4.49 ppm (Fig. 2b,). The integration ratio of the signal at 4.49 to that at 7.81 ppm is 0.92, close to the ideal value (1.0), confirming the integrity of the thiazolidine-2-thione terminal group after polymerization.

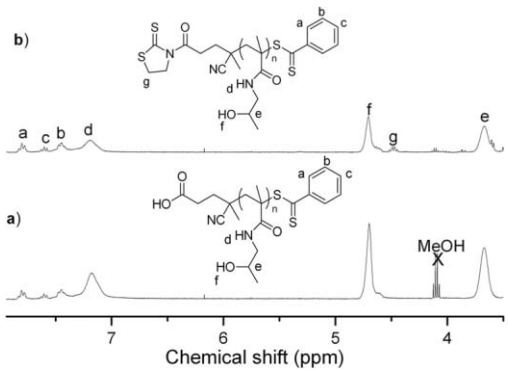


Fig. 2 ^{1}H NMR of polyHPMAs, (a) polymer **1**; (b) polymer **2**.

Protein–polymer conjugation

Polymers with different M_w (polymer **2a**, M_n(NMR) = 3500, PDI = 1.09; polymer **2b**, M_n(NMR) = 6600, PDI = 1.07) were used to modify a protein (lysozyme as a model protein). The reactions were carried out at both pH 6.5 and pH 7.0 with excess polymer (polymer–protein = 40 : 1). GPC was used to characterize the conjugates and the results are shown in Fig. 3a. As the polymer molecular weights increase, the molecular weights of the conjugates also increase. It is noted that when polymer **1** M_n(NMR) = 3600, PDI = 1.09) was mixed with lysozyme, no conjugate was observed.

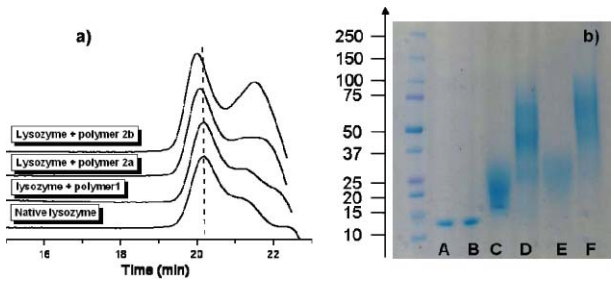


Fig. 3 (a) GPC curves of protein–polymer conjugates at pH 7.0; (b) SDS-PAGE for the protein–polymer conjugates: (A) native lysozyme, (B) lysozyme + polymer **1**, pH 7.0, (C) lysozyme + polymer **2a**, pH 6.5, (D) lysozyme + polymer **2b**, pH 6.5, (E) lysozyme + polymer **2a**, pH 7.0, (F) lysozyme + polymer **2b**, pH 7.0.

SDS-PAGE analysis of the conjugates demonstrated that when polymer **2** was used for conjugation (Fig. 3b), the lysozyme starting material completely disappeared and new bands with higher molecular weights appeared (lane C, D, E, F), consistent with the formation of conjugates. In contrast, there was no conjugation observed when polymer **1** was used under the same conditions (lane B), confirming that the conjugation originates from the reaction between the polymer chain ended thiazolidine-2-thione group and the amine residue on the protein surface. It is evident that the pH of conjugation plays a significant role. At pH 7.0, the resultant conjugates have higher molecular weights (lane E and F) than the corresponding conjugates obtained at pH 6.5 (lane C and D). Therefore more polymer chains are attached to the lysozyme surface at pH 7.0 than at pH 6.5. This phenomenon is ascribed to the fact that the free amine groups will be protonated when the reaction buffer is lower than the amine pK_a and consequently amine nucleophilicity is reduced. Thus it is possible to control the number of polymer chains linked to the protein by adjusting the reaction pH values. This provides a useful design parameter, as the number of chains attached is a crucial factor in determining the activity of the conjugated protein, as described below.

Protein–polymer activity analysis

The activities of lysozyme–polymer conjugates were tested using *Micrococcus lysodeikticus* (Ml) cells as substrates. Protein activity was unaffected (99.3%) when polymer **1** was mixed with protein at pH 6.5 (Fig. 4a) and 7.0 (Fig. 4b), confirming that free polyHPMA does not affect protein activity.

The protein–polymer conjugates exhibited reduced activity when compared to native lysozyme (Fig. 5).

The conjugate with the highest activity (**2b** formed at pH 6.5) yielded an activity of 4.8% compared to native protein. This result can be compared to PEGylated lysozyme, where it has been reported that the bioactivity is totally inhibited with even a single 12 kDa linear PEG fragment.[37] The conjugates formed

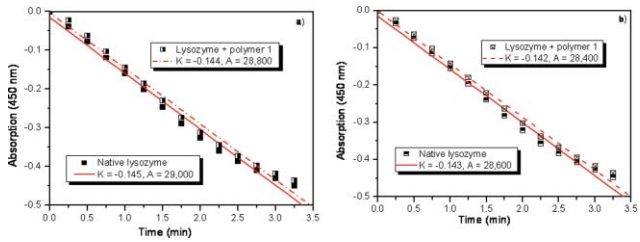


Fig. 4 Bioactivity test of native lysozyme (1.0 mg mL^{-1}, 5 μL) and lysozyme + polymer 1 (5 μL, protein: 1.0 mg mL^{-1}). a) pH 6.5; b) pH 7.0.

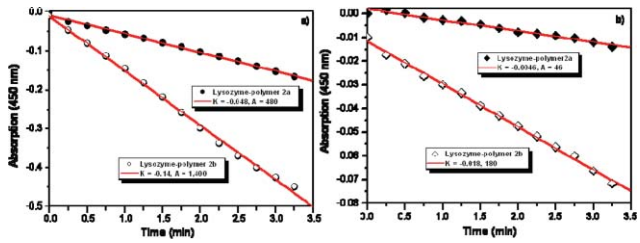


Fig. 5 Activity test of lysozyme–polyHPMA (100 μL, protein: 1.0 mg mL^{-1}). Protein–polymer conjugates obtained at pH 6.5 (a) and at pH 7.0 (b).

at pH 6.5 exhibited almost 10 times more activity than those formed at pH 7.0, which can be attributed to an increased number of polymer chains linked on the protein surface, reducing the interaction between enzyme and substrate. In addition, we note that the bioactivity of conjugates formed at the same pH value show a distinctive molecular weight dependence. Conjugates formed with higher molecular weight polymers display higher bioactivity. This counter-intuitive result can be easily explained with a hypothesis that polymers with higher molecular weights are sterically hindered, reducing the efficiency of coupling to surface amines. The more efficient coupling for lower molecular weight chains, dominates over molecular weight in reducing the protein bioactivity.

Conclusions

In summary, we have described the successful synthesis of new well-defined thiazolidine-2-thione functionalized polyHPMA *via* RAFT polymerization and the subsequent conjugation of the polymer to protein (lysozyme). The molecular weights of the polymers and the pH value of the conjugation reactions affected the bioactivity of the subsequent protein–polymer conjugates. This thiazolidine-2-thione coupling approach is quite general and represents a new, versatile synthetic approach to protein conjugation. When coupled with RAFT polymerization this opens up a wide range of potential polymer–biomolecule conjugates. Currently our work is focused on enhancing the bioactivity of protein–polymer conjugates.

Abbreviations

Chain transfer agent	CTA
Dichloromethane	DCM
Molecular weight	MW
Polydispersity index	PDI
2,2′-Azobis(isobutyronitrile)	AIBN
N,*N*-Dimethylacetamide	DMAc
4-(Dimethylamino)pyridine	DMAP
Gel permeation chromatography	GPC
Molecular weight cut-off	MWCO
N-(2-Hydroxy propyl) methacrylamide	HPMA
Phosphate buffer	PB
Phosphate buffered saline	PBS
Sodium dodecyl sulfate polyacrylamide gel electrophoresis	SDS-PAGE
Micrococcus lysodeikticus	Ml cell

Acknowledgements

TPD thanks Australian Research Council for Federation Fellowship Award. JL acknowledges the UNSW Vice Chancellor's Postdoctoral Research Fellowship.

Notes and references

1 J. M. Harris and R. B. Chess, *Nat. Rev. Drug Discovery*, 2003, **2**, 214–221.
2 M. J. Roberts, M. D. Bentley and J. M. Harris, *Adv. Drug Delivery Rev.*, 2002, **54**, 459–476.
3 F. M. Veronese and J. M. Harris, *Adv. Drug Delivery Rev.*, 2008, **60**, 1–2.
4 R. Langer and J. Folkman, *Nature*, 1976, **263**, 797–800.
5 F. M. Veronese and G. Pasut, *Drug Discovery Today*, 2005, **10**, 1451–1458.
6 R. Duncan, *Nat. Rev. Drug Discovery*, 2003, **2**, 347–360.
7 V. Gaberc-Porekar, I. Zore, B. Podobnik and V. Menart, *Curr. Opin. Drug Discovery Dev.*, 2008, **11**, 242–250.
8 L. Tao, G. Mantovani, F. Lecolley and D. M. Haddleton, *J. Am. Chem. Soc.*, 2004, **126**, 13220–13221.
9 G. Mantovani, F. Lecolley, L. Tao, D. M. Haddleton, J. Clerx, J. J. L. M. Cornelissen and K. Velonia, *J. Am. Chem. Soc.*, 2005, **127**, 2966–2973.
10 F. Lecolley, L. Tao, G. Mantovani, I. Durkin, S. Lautru and D. M. Haddleton, *Chem. Commun.*, 2004, 2026–2027.
11 D. Bontempo, K. L. Heredia, B. A. Fish and H. D. Maynard, *J. Am. Chem. Soc.*, 2004, **126**, 15372–15373.
12 J. Nicolas, V. San Miguel, G. Mantovani and D. M. Haddleton, *Chem. Commun.*, 2006, 4697–4699.
13 D. Bontempo and H. D. Maynard, *J. Am. Chem. Soc.*, 2005, **127**, 6508–6509.
14 K. L. Heredia, D. Bontempo, T. Ly, J. T. Byers, S. Halstenberg and H. D. Maynard, *J. Am. Chem. Soc.*, 2005, **127**, 16955–16960.
15 J. Q. Liu, V. Bulmus, D. L. Herlambang, C. Barner-Kowollik, M. H. Stenzel and T. P. Davis, *Angew. Chem., Int. Ed*, 2007, **46**, 3099–3103.
16 C. Boyer, V. Bulmus, J. Q. Liu, T. P. Davis, M. H. Stenzel and C. Barner-Kowollik, *J. Am. Chem. Soc.*, 2007, **129**, 7145–7154.
17 K. L. Heredia, Z. P. Tolstyka and H. D. Maynard, *Macromolecules*, 2007, **40**, 4772–4779.
18 J. Geng, G. Mantovani, L. Tao, J. Nicolas, G. J. Chen, R. Wallis, D. A. Mitchell, B. R. G. Johnson, S. D. Evans and D. M. Haddleton, *J. Am. Chem. Soc.*, 2007, **129**, 15156–15163.
19 V. Vazquez-Dorbatt and H. D. Maynard, *Biomacromolecules*, 2006, **7**, 2297–2302.
20 A. Lewis, Y. Q. Tang, S. Brocchini, J. W. Choi and A. Godwin, *Bioconjugate Chem.*, 2008, **19**, 2144–2155.
21 D. Samanta, S. Mcrae, B. Cooper, Y. X. Hu and T. Emrick, *Biomacromolecules*, 2008, **9**, 2891–2897.
22 P. De, M. Li, S. R. Gondi and B. S. Sumerlin, *J. Am. Chem. Soc.*, 2008, **130**, 11288.
23 G. Pound, J. M. McKenzie, R. F. M. Lange and B. Klumperman, *Chem. Commun.*, 2008, 3193–3195.
24 L. McDowall, G. J. Chen and M. H. Stenzel, *Macromol. Rapid Commun.*, 2008, **29**, 1666–1671.
25 K. L. Heredia, T. H. Nguyen, C. W. Chang, V. Bulmus, T. P. Davis and H. D. Maynard, *Chem. Commun.*, 2008, 3245–3247.
26 J. Khandare and T. Minko, *Prog. Polym. Sci.*, 2006, **31**, 359–397.
27 G. Pasut and F. M. Veronese, *Prog. Polym. Sci.*, 2007, **32**, 933–961.

28 J. Kopecek, *Ann. N. Y. Acad. Sci.*, 1991, **618**, 335–344.
29 J. Kopecek, P. Kopeckova and C. Konak, *J. Macromol. Sci., Pure Appl. Chem.*, 1997, **34**, 2103–2117.
30 Z. R. Lu, J. G. Shiah, S. Sakuma, P. Kopeckova and J. Kopecek, *J. Controlled Release*, 2002, **78**, 165–173.
31 J. Kopecek, P. Kopeckova, T. Minko and Z. R. Lu, *Eur. J. Pharmaceutics Biopharmaceutics*, 2000, **50**, 61–81.
32 R. Duncan, M. J. Vicent, F. Greco and R. I. Nicholson, *Endocr. Relat. Cancer*, 2005, **12**, S189–S199.
33 K. D. Jensen, P. Kopeckova and J. Kopecek, *Bioconjugate Chem.*, 2002, **13**, 975–984.
34 C. W. Scales, Y. A. Vasilieva, A. J. Convertine, A. B. Lowe and C. L. McCormick, *Biomacromolecules*, 2005, **6**, 1846–1850.
35 Y. Mitsukami, M. S. Donovan, A. B. Lowe and C. L. McCormick, *Macromolecules*, 2001, **34**, 2248–2256.
36 D. Shugar, *Biochim. Biophys. Acta*, 1952, **8**, 302–309.
37 R. B. Greenwald, J. Yang, H. Zhao, C. D. Conover, S. Lee and D. Filpula, *Bioconjugate Chem.*, 2003, **14**, 395–403.