Polymer–Protein Conjugates

DOI: 10.1002/anie.200604922

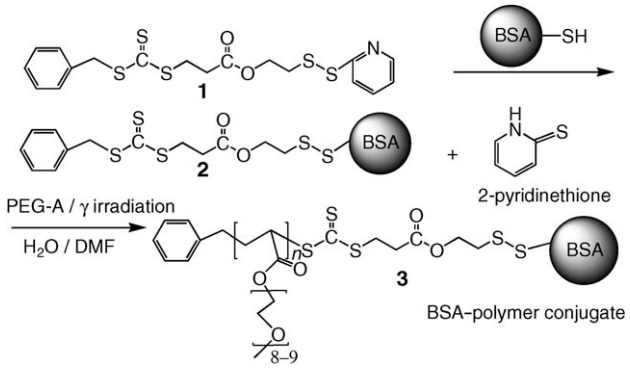
In Situ Formation of Protein–Polymer Conjugates through Reversible Addition Fragmentation Chain Transfer Polymerization**

Jingquan Liu, Volga Bulmus, David L. Herlambang, Christopher Barner-Kowollik, Martina H. Stenzel, and Thomas P. Davis**

Polymer–biomolecule conjugates have attracted increasing interest as a result of their widespread utility in various applications of medicine, biotechnology, and nanotechnology.[1–3] Generally, they have been prepared by postpolymerization conjugation of functionalized polymer chains to biomolecules through covalent and bioaffinity bindings.[4–6] Such postpolymerization conjugation approaches have often involved multiple steps including synthesis, chemical modification, purification, and conjugation. A novel strategy eliminating these multistep procedures has been recently introduced.[7–9] The well-defined polymer–protein conjugates could be prepared in one step by using modified proteins as initiating sites for atom-transfer radical polymerization (ATRP). In situ preparation of polymer–protein conjugates through an ATRP technique has been shown to be advantageous not only for decreasing the number of synthetic steps but also offers the potential of controlling the site and the number of polymer chains conjugated to proteins.

Reversible addition fragmentation chain transfer (RAFT) polymerization has proven to be one of the most versatile controlled/living polymerization techniques.[10–12] The versatility of the RAFT technique to control the polymerization of a wide variety of monomers without using metal catalysts makes it potentially more advantageous than the ATRP technique. RAFT-mediated polymerizations can be performed at room temperature as well as at elevated temperatures in aqueous and organic media.[13–15] In a number of publications, the technique has proven to be useful for the preparation of polymer conjugated systems by in situ polymerization of monomers from the RAFT-agent-anchored substrates.[16–21] However, to our knowledge, the utility of the RAFT technique in the in situ preparation of polymer–biomacromolecule conjugates has not yet been reported.

Herein, we demonstrate the first, RAFT-mediated in situ preparation of polymer–protein conjugates. Adapting the approach used by Maynard and co-workers for preparing a site-specifically modified BSA–ATRP initiator (BSA = bovine serum albumin),[7,8] a new selectively thiol-reactive RAFT agent was synthesized and site-specifically conjugated to BSA through the only free thiol group present on BSA, that is, the cysteine 34 residue. The new RAFT agent, which consists of a trithiocarbonate with a pyridyl disulfide modified Z group and a benzyl R group, is reactive towards the selective exchange reaction with free thiol-tethered molecules under mild conditions.[22] Its reaction with one free thiol group bearing BSA forms a disulfide-linked BSA–RAFT agent conjugate (BSA–macroRAFT agent; Scheme 1). Based on



Scheme 1. Site-specific modification of BSA with a pyridyl disulfide terminated RAFT agent and the polymerization in situ of oligo(ethylene glycol) acrylate (PEG-A). DMF = *N*,*N*-dimethylformamide.

the mechanism of the RAFT polymerization, most of the polymer chains that form during RAFT-mediated polymerization retain a Z-group-attached thiocarbonylthio fragment of the RAFT agent as an end group.[10–12] Therefore, it is reasonable to link BSA at the Z group of a RAFT agent for the RAFT-mediated formation in situ of well-defined BSA–polymer conjugates. The conjugation of a pyridyl disulfide modified RAFT agent to BSA was monitored by using a UV/Vis spectrophotometer, measuring the release of the by-product, 2-pyridinethione, formed during the conjugation reaction (Figure 1). The absorption peak of the conjugation reaction mixture that appeared at approximately 360 nm in the characteristic wavelength range of 2-pyridinethione (approximately 340 nm in aqueous solution and 370 nm in DMF)[22,23] indicated that the conjugation reaction occurred

[*] Dr. J. Liu, Dr. V. Bulmus, D. L. Herlambang, Prof. C. Barner-Kowollik, Dr. M. H. Stenzel, Prof. T. P. Davis
Centre for Advanced Macromolecular Design (CAMD)
School of Chemical Sciences and Engineering
The University of New South Wales
Sydney NSW 2052 (Australia)
Fax: (+61) 2-9385-4749
E-mail: vbulmus@unsw.edu.au
t.davis@unsw.edu.au

[**] V.B. and T.P.D. acknowledge the UNSW Vice Chancellor's Postdoctoral Research Fellowship and the Federation Fellowship Award of the Australian Research Council, respectively. The authors thank the Bioanalytical Mass Spectrometry Facility at UNSW for MALDI analysis, and Dr. L. Barner, Dr. T. Lovestead, and I. Jayencik for their help on the use of the ^{60}Co source.

Supporting information for this article is available on the WWW under http://www.angewandte.org or from the author.

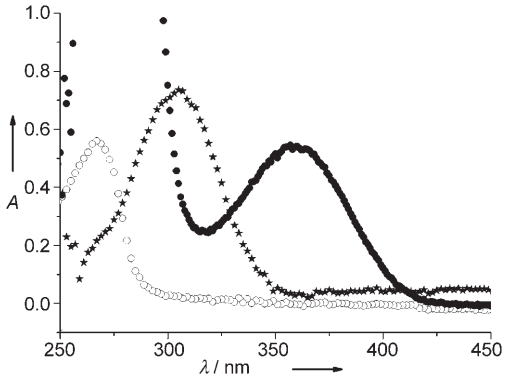


Figure 1. UV/Vis absorption spectra for the reaction of BSA with a modified RAFT agent terminated with a pyridyl disulfide group. The characteristic UV absorption peaks for the initial solutions (after dilution) of BSA and the RAFT agent are also given for comparison (○ BSA; ⋆ RAFT agent; ● after reaction).

through the exchange reaction between the pyridyl disulfide group of the RAFT agent and the free thiol group of BSA. Neither BSA nor the pyridyl disulfide modified RAFT agent absorbs light above 350 nm (Figure 1). The quantification of the conjugation reaction, performed by a colorimetric assay, that is, Ellman's assay,[23] showed that around 44 mol% of BSA could be conjugated with the RAFT agent. It has been previously reported that approximately 50% of cysteine 34 residues from native BSA are present in an oxidized state and therefore only approximately 50 mol% of native BSA is expected to be reactive towards the conjugation with the pyridyl disulfide modified RAFT agent.[7] MALDI-MS analysis of the BSA–RAFT agent conjugate and BSA revealed peaks at masses of 66768 and 66435 amu, respectively (see Figure S1 in the Supporting Information), indicating conjugation of the RAFT agent to the protein. The data obtained with the three different techniques given above confirmed that the BSA–macroRAFT agent was generated successfully.

A water-soluble monomer, oligo(ethylene glycol) acrylate (PEG-A, average molecular weight of 454 g mol^{-1}) was polymerized under γ radiation at room temperature in the presence of the BSA–macroRAFT agent. γ Radiation has been employed previously to initiate RAFT-mediated polymerizations.[24–27] It was reported that there is no difference in the mechanism of the RAFT process between γ ray and thermally initiated polymerizations. The advantage of using γ radiation to initiate a polymerization reaction is that the polymerizations can be performed at room temperature in a variety of solvents including water. However, it has been reported that γ radiation might cause structural damage on biological molecules.[28] A major radiolysis reaction of peptides and proteins in oxygen-free aqueous solutions is intermolecular cross-linking, which leads to the formation of protein aggregates.[28] However, studies have shown that the effect of γ radiation on the structure of proteins is radiation-dose-dependent and can be eliminated completely at a relatively lower radiation flux.[29,30] We conducted our γ ray initiated polymerization experiments in oxygen-free aqueous solutions by using a relatively low γ dose rate, that is, 18.6 Gy h^{-1} via a ^{60}Co γ source. Nevertheless, to rule out the potential effect of γ radiation on protein structure, BSA was incubated in the polymerization solution (the monomer and the RAFT agent with no functional group to attach to BSA under the conditions studied) under γ radiation at a 18.6 Gy h^{-1} dose rate for 15 h. The nondenaturing polyacrylamide gel electrophoresis (PAGE) and MALDI-MS analysis of BSA control samples showed no detectable intermolecular cross-linking reactions or fragmentation caused by the γ radiation at the utilized dose rate (see Figure S2B in the Supporting Information). Furthermore, enzymatic activity of BSA and a relatively more fragile protein, glucose oxidase, after incubation in the polymerization mixture under γ irradiation for 6 h was found to be 92 and 88% of the original activity, respectively (see Figure S3 and S4 in the Supporting Information). As any small alteration in the secondary or tertiary structure of a protein would lead to gross distortions in biological activity, retention of approximately 90% of the original activity rules out the possibility of any major detrimental effect, such as intramolecular interactions, as a result of the γ radiation conditions used in the experiments on the protein structure.

The polymerization of PEG-A was first performed in the presence of both the BSA–macroRAFT agent and the free, pyridyl disulfide modified RAFT agent. The gel-permeation chromatography (GPC) traces of the BSA–macroRAFT agent shifted clearly to higher molecular weight values with increasing polymerization times, indicating the RAFT-mediated in situ formation of BSA–poly(PEG-A) conjugates (Figure 2A). The shoulders on the GPC traces with molecular weights lower than the molecular weight of the BSA–macroRAFT agent indicated the formation of free poly(PEG-A) chains owing to the presence of the free RAFT agent in the polymerization mixture. These free polymer chains that were obviously smaller than the hydrodynamic volume of the BSA–macroRAFT agent could be easily removed by dialysis with a membrane that has a molecular-weight cut off of 50000 Da. The low-molecular-weight tails on the GPC chromatograms of the polymerization mixtures completely disappeared after dialysis (Figure 2B), indicating the removal of the polymer chains formed by the free RAFT-agent-mediated polymerization. The increase in the molecular weight of the BSA–polymer conjugates with increasing polymerization times was clearly observed in the GPC chromatograms. The monomer conversion was found to increase with increasing polymerization time (Figure 2C inset). The molecular weight of the BSA–polymer conjugates determined by MALDI-MS analysis of the dialyzed BSA–polymer conjugates was found to increase linearly with increasing monomer conversions up to approximately 60%. Above 60%, the increase in the molecular weight was much less, which might be due to the effect of increased steric hindrance of growing polymer chains on the accessibility of the RAFT groups.[25,31,32] The nondenaturing PAGE of the dialyzed polymerization mixtures showed clearly the formation of the polymer conjugates with molecular weights higher than the molecular weight of BSA (Figure 2D).

Cleavage of the conjugated polymer from BSA was attempted by reducing the disulfide bond linking the polymer to the protein. However, reducing conditions (1 mM tris(car-

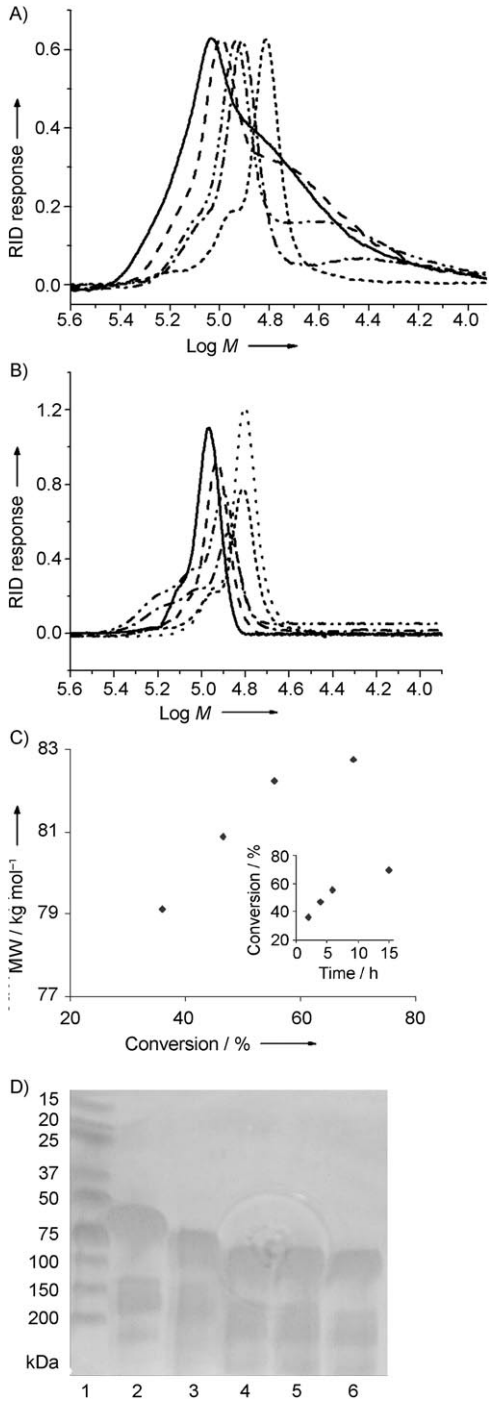


Figure 2. GPC chromatograms of the polymerization mixtures stopped at varying polymerization times, that is, a) before and B) after dialysis (—— 15 h; – – – 6 h; –·–·– 4 h; –•–•– 2 h; ----- BSA–macroRAFT agent; ••••• BSA. *M* = molar mass average of polyethylene oxide and polyethylene glycol standards; RID = refractive index detector. C) Molecular weight (MW) of the dialyzed polymerization mixtures versus the monomer conversion (MW data obtained from mass spectrometry analysis). Inset: Monomer conversion versus polymerization time. D) Nondenaturing PAGE of the dialyzed polymerization mixtures: Lane 1 = molecular weight markers, lane 2 = BSA–macroRAFT agent, lanes 3–6 = dialyzed polymerization mixtures with increasing monomer conversions, respectively.

boxyethyl)phosphine (TCEP) for 2 h), which were mild enough not to disturb the structural integrity of BSA, were found not to cleave the polymer chains from the BSA–higher-molecular-weight conjugates (above 80000 Da). Under the same reducing conditions, partial cleavage of the polymer chains from BSA was observed only with the low-molecular-weight conjugate (data not shown). This observation might be explained by the partial or total embedding of the disulfide bond that links the polymer chain to the protein, which decreases the accessibility of the bond by the reducing agent. A slight increase in the polymeric chain length might cause a more profound shielding effect on the protein owing to the repeating side oligo(ethylene glycol) units, therefore the cleavage of the disulfide bond linking the polymer to the protein might be more difficult in the case of higher-molecular-weight polymer conjugates. When stronger reducing conditions (100 mM TCEP, 24 h) were utilized, BSA was completely reduced into peptide fragments with molecular weights of less than 10000 Da, revealing the free polymer chains. The GPC analysis of these samples in which BSA was completely cleaved allowed the molecular weight and the molecular-weight distribution of the polymer chains, which were generated from the conjugate of BSA, to be determined (Figure 3 A and B). The linear evolution of the molecular weight of the polymers with increasing monomer conversions up to approximately 70 % evidenced further the occurrence of

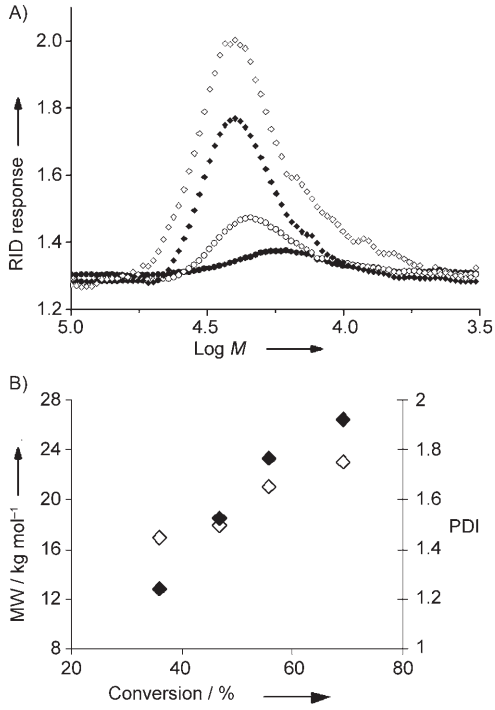


Figure 3. BSA–polymer conjugates after the complete cleavage of BSA with TCEP (100 mM) for 24 h. A) GPC chromatograms (● 2 h; ○ 4 h; ◆ 6 h; ◇ 15 h) and B) the evolution of the number-average molecular weight (◆) and the molecular weight distribution (PDI; ◇) with monomer conversion, as determined by GPC and ^{1}H NMR spectroscopy. The samples contain the free polymer chains cleaved from BSA and also the small-molecular-weight oligopeptide products formed upon the complete cleavage of BSA.

the RAFT-mediated polymerization in situ. The large polydispersity index (PDI) values of the samples might be due to the hindrance of the RAFT end groups by the growing polymer chain on the protein.[25,31,32] Furthermore, the presence of low-molecular-weight peptide fragments formed by the cleavage of BSA might have also caused the lower-molecular-weight tails on GPC traces and hence increased the PDI values.

A control experiment performed with a mixture of BSA, RAFT agent without a pyridyl disulfide group, and PEG-A monomer incubated under γ irradiation for 15 h, indicated the formation of a physical mixture of the free BSA and the free polymer, but not the polymer–BSA conjugate (see Figure S2 in the Supporting Information). This control experiment further evidenced that the in situ polymerization could take place at the site of the RAFT agent conjugated with BSA.

We further checked whether the polymerization in situ of PEG-A could be controlled by the BSA–macroRAFT agent in the absence of the free RAFT agent. The major peak observed on the GPC chromatograms of the polymerization mixtures indicated the formation of macromolecules with hydrodynamic volumes larger than that of BSA (Figure 4A). The peaks were found to be shifted to higher molecular weights with increasing polymerization times. The low-molecular-weight tail on the chromatograms might be mainly associated with the existence of the free BSA that was not conjugated with a RAFT agent and also the formation of free polymer chains during polymerization. The extent of the formation of the free polymers with a molecular weight lower than the molecular weight of BSA was less significant compared with the formation of the low-molecular-weight free polymer in the polymerizations performed in the presence of the free RAFT agent. The inhibition period of two hours observed in the polymerization might be associated with the slow fragmentation of the intermediate BSA–macroRAFT agent (Figure 4b, inset). The linear evolution of the molecular weight with increasing monomer conversion revealed that the in situ formation of the protein conjugates was controlled by the RAFT mechanism. The PDI values that do not exactly reflect the true molecular-weight distribution of the polymeric chains grafted to BSA might be associated with the reduced accessibility of the RAFT end groups owing to the steric hindrance by the polymer chains.[25,31,32]

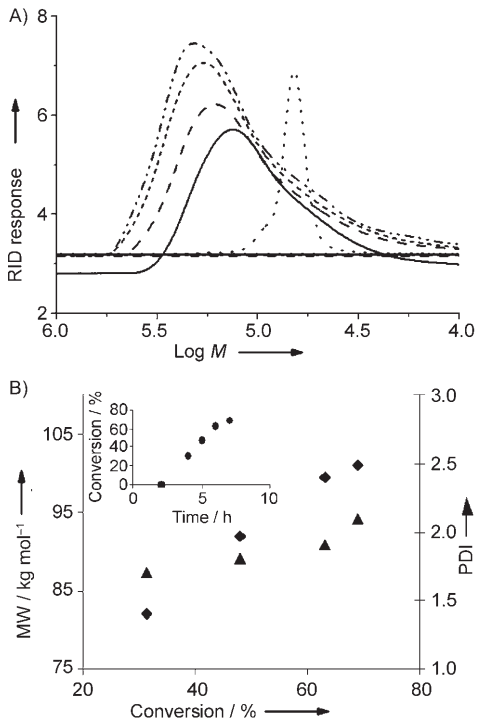


Figure 4. The molecular weight and conversion evolution during the polymerization in situ without the free RAFT agent. A) GPC chromatograms of the polymerization mixtures stopped at varying polymerization times: –·–· 7 h; ----- 6 h; – – – 5 h; —— 4 h; ••••• BSA. B) The number-average molecular weights determined by GPC versus the monomer conversion determined by ^{1}H NMR spectroscopy. The inset shows the monomer conversion versus polymerization time (◆ molecular weight; ▲ polydispersity index (PDI)).

In conclusion, the first, RAFT-mediated in situ preparation of protein–polymer conjugates was presented. A new biohybrid RAFT agent, that is, the BSA–macroRAFT agent was prepared and used to control the γ ray initiated polymerization of a water-soluble monomer at room temperature. The in situ formation of the polymer chains was found to take place at the site of the RAFT agent conjugated to BSA and in the solution when the free RAFT agent was used in conjunction with the BSA–macroRAFT agent. The polymerization performed without the free RAFT agent yielded the formation of BSA–polymer conjugates along with the relatively less significant free-polymer generation. The linear evolution of molecular weights with monomer conversions indicated that the in situ polymerizations were controlled by the RAFT mechanism. Our future efforts will be focused on the exploitation of the RAFT technique on in situ preparation of functional polymer–biomolecule conjugates.

Experimental Section

The synthesis of the pyridyl disulfide terminated RAFT agent was given elsewhere.[22] All other experiments conducted in the study are described in the Supporting Information.

Received: December 6, 2006
Published online: March 20, 2007

Keywords: polymer conjugates · polymerization · protein conjugates · radiation

[1] D. W. Pack, A. S. Hoffman, S. Pun, P. S. Stayton, *Nat. Rev. Drug Discovery* **2005**, *4*, 581 – 593.
[2] J. M. Harris, R. B. Chess, *Nat. Rev. Drug Discovery* **2003**, *2*, 214 – 221.
[3] R. Duncan, *Nat. Rev. Drug Discovery* **2003**, *2*, 347 – 360.
[4] L. Tao, G. Mantovani, F. Lecolley, D. M. Haddleton, *J. Am. Chem. Soc.* **2004**, *126*, 13220 – 13221.
[5] V. Bulmus, Z. L. Ding, C. J. Long, P. S. Stayton, A. S. Hoffman, *Bioconjugate Chem.* **2000**, *11*, 78 – 83.
[6] J. M. Hannink, J. J. L. M. Cornelissen, J. A. Farrera, P. Foubert, F. C. De Schryver, N. A. J. M. Sommerdijk, R. J. M. Nolte,

Angew. Chem. **2001**, *113*, 4868 – 4870; *Angew. Chem. Int. Ed.* **2001**, *40*, 4732 – 4734.
[7] K. L. Heredia, D. Bontempo, T. Ly, J. T. Byers, S. Halstenberg, H. D. Maynard, *J. Am. Chem. Soc.* **2005**, *127*, 16955 – 16960.
[8] D. Bontempo, K. L. Heredia, B. A. Fish, H. D. Maynard, *J. Am. Chem. Soc.* **2004**, *126*, 15372 – 15373.
[9] B. S. Lele, H. Murata, K. Matyjaszewski, A. J. Russell, *Biomacromolecules* **2005**, *6*, 3380 – 3387.
[10] C. Barner-Kowollik, T. P. Davis, J. P. A. Heuts, M. H. Stenzel, P. Vana, M. Whittaker, *J. Polym. Sci. Part A* **2003**, *41*, 365 – 375.
[11] G. Moad, E. Rizzardo, S. H. Thang, *Aust. J. Chem.* **2005**, *58*, 379 – 410.
[12] S. Perrier, P. Takolpuckdee, *J. Polym. Sci. Part A* **2005**, *43*, 5347 – 5393.
[13] J. F. Quinn, L. Barner, C. Barner-Kowollik, E. Rizzardo, T. P. Davis, *Macromolecules* **2002**, *35*, 7620 – 7627.
[14] C. W. Scales, Y. A. Vasilieva, A. J. Convertine, A. B. Lowe, C. L. McCormick, *Biomacromolecules* **2005**, *6*, 1846 – 1850.
[15] C. M. Schilli, M. F. Zhang, E. Rizzardo, S. H. Thang, Y. K. Chong, K. Edwards, G. Karlsson, A. H. E. Muller, *Macromolecules* **2004**, *37*, 7861 – 7866.
[16] C. Li, J. Han, C. Y. Ryu, B. C. Benicewicz, *Macromolecules* **2006**, *39*, 3175 – 3183.
[17] L. Barner, N. Zwaneveld, S. Perera, Y. Pham, T. P. Davis, *J. Polym. Sci. Part A* **2002**, *40*, 4180 – 4192.
[18] F. D'Agosto, M. T. Charreyre, C. Pichot, R. G. Gilbert, *J. Polym. Sci. Part A* **2003**, *41*, 1188 – 1195.
[19] M. G. J. ten Cate, H. Rettig, K. Bernhardt, H. G. Börner, *Macromolecules* **2005**, *38*, 10643 – 10649.
[20] M. Bathfield, F. D'Agosto, R. Spitz, M. Charreyre, T. Delair, *J. Am. Chem. Soc.* **2006**, *128*, 2546 – 2547.
[21] C. Y. Hong, C. Y. Pan, *Macromolecules* **2006**, *39*, 3517 – 3524.
[22] J. Liu, V. Bulmus, C. Barner-Kowollik, M. Stenzel, T. P. Davis, *Macromol. Rapid Commun.* **2007**, 28, 305 – 314.
[23] G. T. Hermanson, *Bioconjugate Techniques*, Academic Press, New York, **1996**.
[24] J. F. Quinn, L. Barner, E. Rizzardo, T. P. Davis, *J. Polym. Sci. Part A* **2002**, *40*, 19 – 25.
[25] P. E. Millard, L. Barner, M. H. Stenzel, T. P. Davis, C. Barner-Kowollik, A. H. E. Muller, *Macromol. Rapid Commun.* **2006**, *27*, 821 – 828.
[26] L. Barner, J. F. Quinn, C. Barner-Kowollik, P. Vana, T. P. Davis, *Eur. Polym. J.* **2003**, *39*, 449 – 459.
[27] T. Lovestead, G. Hart-Smith, T. P. Davis, M. H. Stenzel, C. Barner-Kowollik, unpublished results .
[28] W. M. Garrison, *Chem. Rev.* **1987**, *87*, 381 – 398.
[29] T. Kume, T. Matsuda, *Radiat. Phys. Chem.* **1995**, *46*, 225 – 231.
[30] Y. Lee, K. B. Song, *J. Biochem. Mol. Biol.* **2002**, *35*, 590 – 594.
[31] R. Wang, C. L. McCormick, A. B. Lowe, *Macromolecules* **2005**, *38*, 9518 – 9525.
[32] M. H. Stenzel, T. P. Davis, *J. Polym. Sci. Part A* **2002**, *40*, 4498 – 4512.

