Application of the Biodegradable Diblock Copolymer Poly(L-lactide)-*block*-Poly(L-cysteine): Drug Delivery and Protein Conjugation

Jing Sun,[1,2] Xuesi Chen,[1] Jizheng Wei,[1,2] Lesan Yan,[1,2] Xiabin Jing[1]

[1]*State Key Laboratory of Polymer Physics and Chemistry, Changchun Institute of Applied Chemistry, Chinese Academy of Sciences, Changchun 130022, People's Republic of China*
[2]*Graduate School, Chinese Academy of Sciences, Beijing 100049, People's Republic of China*

Received 23 June 2009; accepted 21 February 2010
DOI 10.1002/app.32341
Published online 7 June 2010 in Wiley InterScience (www.interscience.wiley.com).

ABSTRACT: A novel approach to self-assembled and shell-crosslinked (SCL) micelles from the diblock copolymer poly(L-lactide)-*block*-poly(L-cysteine) to be used as drug and protein delivery carriers is described. Rifampicin was used as a model drug. The drug-loaded SCL micelles were obtained by self-assembly of the copolymer in the presence of the drug in aqueous media. Their morphology and size were studied with dynamic light scattering and field emission scanning electron microscopy. The rifampicin loading capacity and encapsulation efficiency were studied with ultraviolet–visible spectrophotometry. The drug-release rate *in vitro* depended on the oxidizing and reducing environment. Moreover, a straightforward approach to the conjugation of the copolymer with bovine serum albumin (BSA) was developed, and a gel electrophoresis test demonstrated that this conjugated BSA could be reversibly released from the copolymer substrate under reducing conditions. In conclusion, this L-cysteine copolymer can be used in drug delivery and in protein fixation and recovery. © 2010 Wiley Periodicals, Inc. J Appl Polym Sci 118: 1738–1742, 2010

Key words: block copolymers; crosslinking; self-assembly

INTRODUCTION

In the past 3 decades, biodegradable polymers have become more and more important in pharmaceutical and biomedical fields.[1,2] Among them, polylactide, a very important synthetic biodegradable material, has been widely used in surgical repair, in carriers for drug delivery, and in temporary matrices or scaffolds for tissue engineering[3–5] because of its biodegradability, biocompatibility, high mechanical properties, and excellent shaping and molding properties. Its block copolymerization with peptides can modify its degradation pattern because peptidase is required to hydrolyze the peptide bonds. Moreover, polypeptide, a natural material, is a good choice for the design of novel biomaterials. Compared with those block copolymers without polypeptide blocks, polypeptide-containing ones can simulate not only the shape of natural aggregates but also their biological performance. However, only a few biodegradable copolymers consisting of aliphatic polyester and polypeptide chains have been studied so far.[4–10]

Amphiphilic block copolymers have been researched extensively in recent years.[11–13] In aqueous media, these copolymers can spontaneously form micelles, which have a kind of core–shell architecture. The hydrophobic segments of the copolymers segregate into the core, which is protected by the shell from the external media, whereas the hydrophilic segments of the copolymer form the shell. This kind of core–shell architecture is widely used in pharmaceutical applications.[14–18] The hydrophobic core serves as a nanocontainer for hydrophobic drugs, whereas the hydrophilic shell increases the micelle stability and protects the system from the environmental reticuloendothelial system uptake.[19] Many factors can influence the drug-loading capacity and release kinetics: the length of the core and shell-forming segments in the copolymer, the physical and chemical properties of the drug and the core-forming hydrophobic segments, the compatibility between the two, and so forth. Furthermore, an important consideration in drug-delivery applications is that the copolymer micelles will dissociate into unimers when the copolymer concentration falls below the critical

Correspondence to: X. Jing (xbjing@ciac.jl.cn).
Contract grant sponsor: National Natural Science Foundation of China; contract grant numbers: 20674084 and 20774093.
Contract grant sponsor: National Fund for the Distinguished Young Scholars; contract grant number: 50425309.
Contract grant sponsor: Ministry of Science and Technology of China through the 973 Project; contract grant number: 2009CB930102.

micelle concentration with dilution under physiological conditions, and this will result in prerelease of the encapsulated drugs.[20] However, this problem can be overcome with shell-crosslinked (SCL) micelles.[21] To date, several methods have been employed for the formation of SCL micelles.[22,23] Recently, we synthesized a new diblock copolymer, poly(L-lactide)-*block*-poly(L-cysteine) (PLLA-*b*-PLC), by the ring-opening polymerization (ROP) of *N*-carboxyanhydride of β-benzyloxycarbonyl-L-cysteine (ZLC–NCA) with amino-terminated poly(L-lactide) (PLLA–NH_2) as a macroinitiator, and from it, reversible SCL spherical micelles were obtained in aqueous media.[24] In this study, the model drug rifampicin was encapsulated into SCL micelles, and its release was tested *in vitro*. Moreover, the thiol groups that formed after reduction in the copolymer PLLA-*b*-PLC were used to combine a model protein: bovine serum albumin (BSA). After its reaction with dithiothreitol (DTT), the escape of BSA from the copolymer was observed, and this demonstrated the reversible conjugation of BSA with PLLA-*b*-PLC. This can be used in medicine and biotechnology.[25,26]

EXPERIMENTAL

Materials

The PLLA-*b*-PLC copolymers were reduction products of poly(L-lactide)-*block*-poly(*S*-benzyloxycarbonyl-L-cysteine) and were synthesized via ROP of ZLC–NCA initiated by PLLA–NH_2, as reported by Jing et al.[24] Rifampicin (a drug for tuberculosis) was a commercial product (Shanghai, China). BSA (96%) was purchased from Sigma, USA. DTT was purchased from Beijing Xiasi Biotech Co., Ltd. (Beijing, China).

Rifampicin loading into block copolymer micelles

The SCL micelles containing rifampicin were prepared according to ref. 24. Rifampicin (15 mg) and the block copolymer PLLA-*b*-PLC (15 mg) were dissolved together in 10 mL of dimethylformamide. Then, 50 equiv of DTT with respect to the thiol groups was added, and the mixture was stirred for 2 h at room temperature. Then, a given amount of deionized water was added to the copolymer solution with gentle stirring. To reach equilibrium, the mixture was stirred overnight. After that, the mixture was further diluted with a large amount of deionized water. Then, it was dialyzed against a phosphate buffer (pH 6.8) to form SCL micelles and against deionized water to remove the salt and free rifampicin and DTT. The drug content in the micellar solutions was determined at 480 nm with an ultraviolet–visible (UV–vis) spectrophotometer (UV-2401PC, Shimadzu, Japan). After freeze drying, the rifampicin-loaded micelles were stored at −20°C until use. The drug-loading content and entrapment efficiency were defined as the weight percentages of the drug in the micelles with respect to the total weight of the micelles and with respect to the total weight of the drug fed initially, respectively.

Dynamic light scattering (DLS) measurements

DLS measurements were carried out with a Dawn EOS instrument (Wyatt Technology, USA) equipped with a He–Ne laser with a 632.8-nm wavelength at a scattering angle of 90°. A rifampicin-loaded micelle solution (ca. 0.4 mg/mL) was passed through a 0.45-μm filter before measurement.

Environmental scanning electron microscopy (ESEM) measurements

The ESEM images were recorded with an XL 30 field emission gun scanning electron microscope from Micro FEI Philips, USA. A rifampicin-loaded micelle solution was deposited onto a silicon wafer to form a very thin layer and was dried at room temperature. A thin layer of gold was coated onto the sample surface before measurement.

In vitro rifampicin release from block copolymer micelles

In vitro release profiles of the rifampicin micelles were investigated in phosphate-buffered saline (PBS; pH = 7.4). The weighed and freeze-dried rifampicin micelles were suspended in a 4-mL PBS solution in a dialysis membrane bag (molecular weight cutoff = 3500 Da). The release experiment was initiated by the placement of the end-sealed dialysis bag in a 16-mL PBS solution at 37°C with continuous shaking at 70 rpm to measure the released rifampicin from the micelles. At a certain interval, samples (4 mL) were withdrawn, and then the same volume of a fresh buffer medium was added. The amount of released rifampicin in the collected medium was measured at 472 nm with a UV–vis spectrophotometer (UV-2401PC, Shimadzu). All experiments were done in duplicate.

Conjugation of BSA to PLLA-*b*-PLC

The block copolymer (5 mg) was dissolved in 0.5 mL of DMSO. Then, 50 equiv of DTT with respect to the thiol groups was added under stirring. A solution of BSA [1 mg of BSA/0.25 mL of phosphate buffer (pH = 8.0)] was added to the copolymer solution with gentle stirring. After 12 h, it was dialyzed against the phosphate buffer (pH 8.0) and then stored at 4°C until use. Sodium dodecyl

sulfate/polyacrylamide gel electrophoresis (SDS–PAGE) was performed with 10% polyacrylamide gels. The samples were heated at 100°C for 5 min after vortexing with a given amount of a sodium dodecyl sulfate solution. Staining was accomplished with Coomassie blue. For reducing conditions, DTT was freshly added.

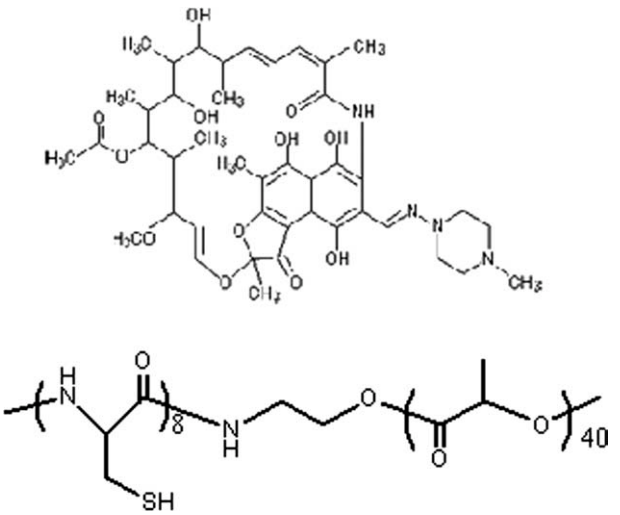
RESULTS AND DISCUSSION

Synthesis and characterization of the diblock copolymers

Recently, Jing et al.[24] reported on the synthesis of the novel diblock copolymer PLLA-*b*-PLC. First, PLLA–NH_2 was obtained by ROP of L-lactide with stannous octoate as the catalyst and 2-benzyloxycarbonyl aminoethanol as the initiator and by subsequent deprotection. The second block was synthesized by ROP of *N*-carboxyanhydride of *S*-benzyloxycarbonyl-L-cysteine with PLLA–NH_2 as the macroinitiator and by subsequent deprotection with HBr. In this study, the same procedure was adapted to prepare the diblock copolymer PLLA(40)-*b*-PLC(8); the numbers in parentheses designate degrees of polymerization of the two blocks (Scheme 1). As shown in ref. 24, this copolymer can self-assemble into spherical micelles in an aqueous medium with poly(L-lactide) (PLLA) segments in the core and poly(L-cysteine) (PLC) segments in the corona; most of the thiol groups exist in disulfide form because of oxidation by air, and this leads to shell crosslinking of the micelles. On the other hand, this shell crosslinking can be destroyed by the addition of enough DTT. This reversible conversion from thiol groups to disulfide bonds was used in this study to control the drug release and trigger the protein fixation and release, as shown in the following sections.

Preparation and characterization of the rifampicin-loaded SCL micelles

It has been reported that the kinetic stability of polymer micelles is greatly improved by the formation of crosslinked shells.[21] These biodegradable, water-soluble colloids are less prone to aggregation during the lyophilization process, and the dried particles are recovered as polymer micelles from the aqueous solution; therefore, they have attractive potential for drug-release systems.[21] In this study, nanoscale core–shell-type polymeric micelles of the diblock copolymer PLLA(40)-*b*-PLC(8) were prepared in the presence of a model drug with a normal micelle preparation procedure, as described in the Experimental section. Rifampicin (Scheme 1) was used as a model compound for controlled-release studies.



Scheme 1 Chemical structures of rifampicin and its carrier polymer.

Rifampicin could be easily encapsulated in the hydrophobic core because of its hydrophobic interaction with PLLA segments. The drug-loading content and entrapment efficiency of the polymeric micelles were 15.0% and 17.5%, respectively. The morphology of the rifampicin-loaded polymeric micelles was observed by ESEM [Fig. 1(B)]. The nanoparticles were also spherical in shape, and their mean diameter was about 65 nm. The size distribution of the nanoparticles was measured by DLS [Fig. 1(D)]. The hydrodynamic radius (R_h) of the rifampicin-loaded polymeric micelles was 66.6 nm, which was a little larger than that of the polymeric micelles without rifampicin [47.1 nm; Fig. 1(C)]. The particle diameters measured by ESEM were a little smaller than the hydrodynamic diameters measured by DLS. This may be due to the volume shrinkage during sample drying. A few aggregates were found in the rifampicin-loaded micelles by ESEM [Fig. 1(B)]. They probably formed during sample drying because the micelles were not stable enough on account of the increased hydrophobicity of the micellar cores caused by rifampicin incorporation.

In vitro drug-release behavior

The rifampicin release behavior from the SCL micelles *in vitro* was examined in PBS (0.1 mol/L, pH 7.4) at 37°C with a conventional dialysis method. To demonstrate the effect of shell crosslinking in the micelles, release experiments were performed in the presence and absence of DTT; that is, 50 equiv of DTT with respect to the thiol groups was added to the micelle solution 2 h before the beginning of the release experiment for the former case, and nothing was added in the latter case. Because DTT could reduce disulfide bonds into thiol groups, the

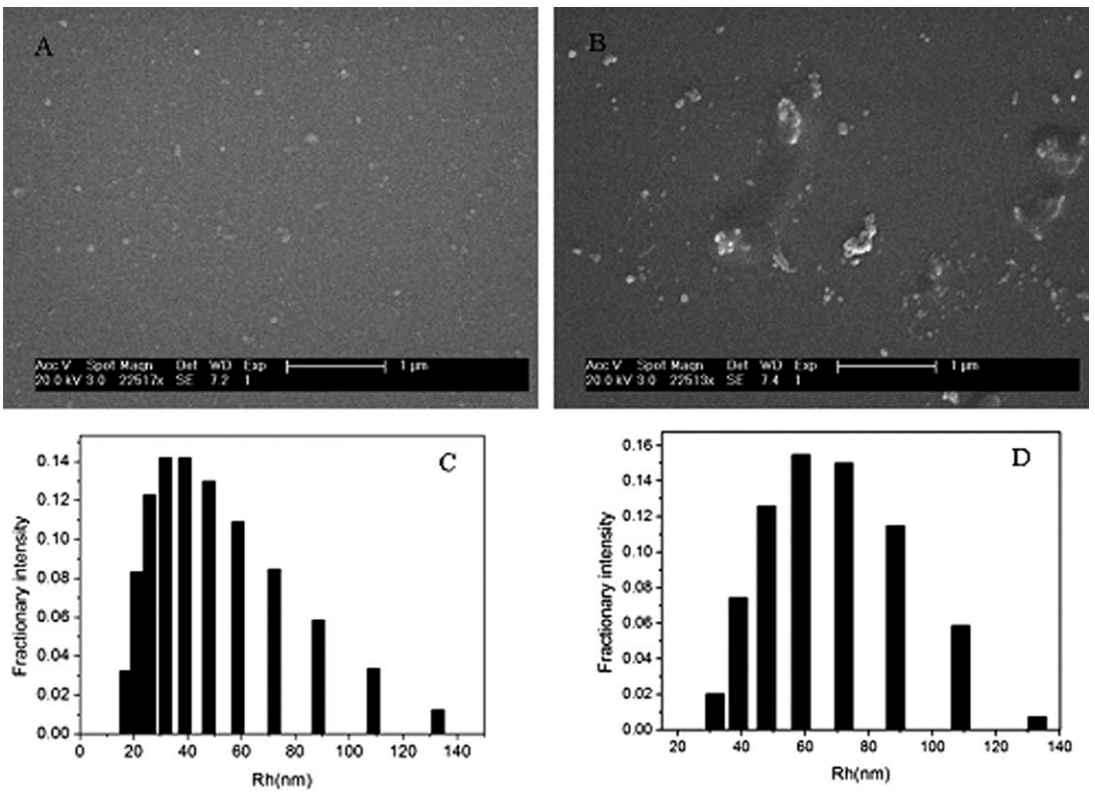


Figure 1 (A) ESEM micrograph of the SCL micelles prepared in H_2O, (B) ESEM micrograph of the micelles containing rifampicin, (C) DLS graph of the micelles prepared in H_2O, and (D) DLS graph of the micelles containing rifampicin.

crosslinking in the micelles in the presence of DTT was considered to have been destroyed.[24] As shown in Figure 2, the two samples showed similar release kinetics, that is, faster release in the initial stage and slower release in the later stage. However, the release rate was higher in the presence of DTT than in the absence of DTT. After 24 h, the rifampicin release percentage of the SCL micelles was about 50%, whereas it was about 90% in the presence of DTT. This difference can be explained by the elimination of the shell crosslinking of the micelles in the presence of DTT: the drug molecules could diffuse faster from a noncrosslinked micelle to the phosphate buffer than from an SCL micelle.

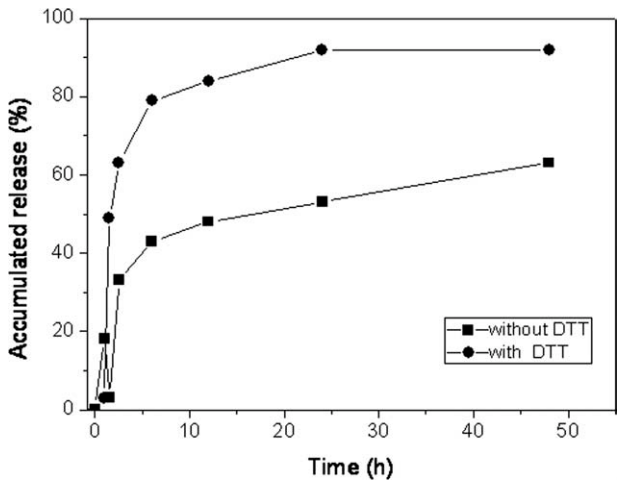


Figure 2 *In vitro* release of rifampicin from the polymeric micelles in the presence and absence of DTT.

Conjugation of BSA to PLLA-*b*-PLC

Because of the existence of the pendent thiol groups on the PLC segments, the copolymers could react with proteins to form bioconjugates. To demonstrate this, BSA, a globular protein possessing 17 disulfide bonds and a free cysteine as residue 34, was used as a model protein. Because oxygen in the air could oxidize the free thiol groups on the copolymer into disulfide bonds, the copolymer was reduced with DTT first to make the thiol groups available, and

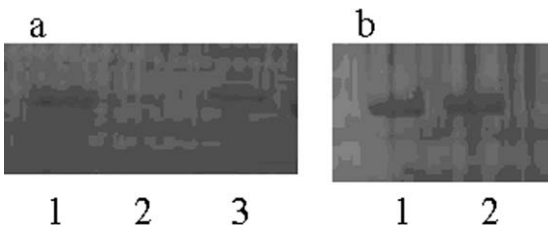


Figure 3 SDS–PAGE under (a) reducing and (b) nonreducing conditions: (a1,b1) pure BSA; (a2) BSA and reduced copolymer; (a3) BSA, reduced copolymer; and DTT; and (b2) BSA and nonreduced copolymer.

then it was incubated with BSA. Formation of the conjugates was verified by gel electrophoresis [Fig. 3(a)]. The conjugates (lane a2) showed no bar comparable to free BSA (lane a1). However, when this system was treated with DTT (lane a3), an identical bar with free BSA was observed. This can be described as follows. After the conjugation of BSA to the reduced copolymers followed by dialysis, the formed conjugates precipitated from the aqueous media, mainly because of the hydrophobic feature of the PLLA segments. The precipitated micelles were not movable in the gel plate, and so nothing was observed at the position of free BSA (lane a2). When the reducing agent DTT was added to the system, the conjugates were destroyed, and free BSA was released; this yielded the same gel electrophoresis bar as free BSA (lane a3).

To exclude the possibility of simply mixing BSA and the copolymer, BSA was incubated with the nonreduced copolymer. As expected, the same band found for the free BSA was obtained (lane b2). This result showed that the coupling of the copolymer and BSA did not occur because the free thiol groups were not available in the nonreduced copolymer.

In summary, these SDS–PAGE results have shown that under reducing conditions, BSA can be conjugated to the PLLA-*b*-PLC copolymer, and by dialysis, the unreacted BSA can be removed completely; the conjugated BSA can be released by the addition of DTT. Although we cannot know whether BSA is attached to the copolymer chain at cysteine residue 34, it is safe to say that the conjugation occurs through a reversible disulfide bond between the PLC segment and the BSA molecule.

CONCLUSIONS

Diblock copolymer micelles containing the model drug rifampicin or the model protein BSA were prepared from a novel diblock copolymer (PLLA-*b*-PLC) that could self-assemble into SCL micelles in an aqueous solution because of the oxidative coupling of the thiol groups on the PLC segments. The disulfide bonds that formed could be reduced to thiol groups with DTT. Because of this reversibility between thiol groups and disulfide bonds, the SCL micelles could be used to encapsulate drugs or conjugate proteins. The drug or protein release could be accelerated by the reduction of the disulfide bonds. Therefore, this copolymer could be used in drug delivery or in protein fixation and recovery. Because there are glutathione and related enzymes in the cytoplasm, they can serve as reducing agents for the disulfide bonds and accelerate the intracellular release of drugs or proteins. Further investigations are underway, and related results will be published elsewhere.

References

1. Gombotz, W. R.; Pettit, D. K. Bioconjugate Chem 1995, 6, 51.
2. Ryser, H. G. P.; Shen, W. C. Proc Natl Acad Sci USA 1978, 75, 70.
3. Zeng, J.; Xu, X. Y.; Chen, X. S.; Liang, Q. Z.; Bian, X. C.; Yang, L. X.; Jing, X. B. J Controlled Release 2003, 92, 31.
4. Holland, S. J.; Gould, B. J. J Controlled Release 1986, 4, 80.
5. Gilding, D. K.; Reed, A. M. Polymer 1979, 20, 64.
6. Kros, A.; Jesse, W.; Metselaar, G. A.; Cornelissen, J. J. L. M. Angew Chem Int Ed 2002, 8, 41.
7. Nowak, A. P.; Breedveld, V.; Pakstis, L.; Ozbas, B.; Pine, D. J.; Pochan, D.; Deming, T. J. Nature 2002, 417, 424.
8. Mao, C. B.; Solis, D. J.; Reiss, B. D.; Kottmann, S. T.; Sweeney, R. Y.; Haryhurst, A.; Georgiou, G.; Iverson, B.; Belcher, A. M. Science 2004, 303, 213.
9. Arimura, H.; Ohya, Y.; Ouchi, T. Biomacromolecules 2005, 6, 720.
10. Li, Y.; Li, Q.; Li, F.; Zhang, H.; Jia, L.; Yu, J.; Fang, Q.; Cao, A. Biomacromolecules 2006, 7, 224.
11. Gao, Z.; Eisenberg, A. Macromolecules 1993, 26, 7353.
12. Won, Y. Y.; Davis, H. T.; Bates, F. S. Science 1999, 283, 960.
13. Forster, S.; Plantenberg, T. Angew Chem Int Ed 2002, 41, 688.
14. Bishara, A.; Kricheldorf, H. R.; Domb, A. J. Macromol Symp 2005, 225, 17.
15. Kwon, G. S.; Okano, T. Adv Drug Delivery Rev 1996, 21, 107.
16. Hagan, S. A.; Coombes, A. G. A.; Garnett, M. C.; Dunn, S. E.; Davies, M. C.; Illum, L.; Davis, S. S.; Harding, S. E.; Purkiss, S.; Gellert, P. R. Langmuir 1996, 12, 2153.
17. Jeong, B.; Bae, Y. H.; Lee, D. S.; Kim, S. W. Nature 1997, 388, 860.
18. Langer, R. Science 2001, 293, 58.
19. Gref, R.; Minamitake, Y.; Peracchia, M. T.; Trubetskoy, V.; Torchilin, V.; Langer, R. Science 1994, 263, 1600.
20. Bronich, T. K.; Keifer, P. A.; Shlyakhtenko, L. S.; Kabanov, A. V. J Am Chem Soc 2005, 127, 8236.
21. Thurmond, K. B.; Kowalewski, T.; Wooley, K. L. J Am Chem Soc 1996, 118, 7239.
22. Weaver, J. V. M.; Tang, Y. Q.; Liu, S.; Iddon, P. D.; Grigg, R.; Armes, S. P.; Billingham, N. C.; Hunter, R.; Rannard, S. P. Angew Chem Int Ed 2004, 43, 1389.
23. Li, Y.; Lokitz, B.; McCormick, C. L. Macromolecules 2005, 39, 81.
24. Sun, J.; Chen, X. S.; Lu, T. C.; Liu, S.; Tian, H. Y.; Guo, Z.; Jing, X. B. Langmuir 2008, 24, 10099.
25. Duncan, R. Nat Rev Drug Discovery 2003, 2, 347.
26. Hoffman, A. S.; Stayton, P. S. Macromol Symp 2004, 207, 139.