Covalent Immobilization of an Antimicrobial Peptide on Poly(ethylene) Film

Matthew D. Steven, Joseph H. Hotchkiss

Cornell Institute of Food Science, Department of Food Science, Stocking Hall, Cornell University, Ithaca, New York 14853

Received 22 March 2007; accepted 7 November 2007
DOI 10.1002/app.27638
Published online 26 August 2008 in Wiley InterScience (www.interscience.wiley.com).

ABSTRACT: An antimicrobial film was produced by covalently attaching synthetic peptide E14LKK to poly(ethylene) film. E14LKK is a 14 residue, magainin-class peptide with broad-spectrum antimicrobial activity. The poly(ethylene) surface was first oxidized with chromic/sulphuric acid, then PEGylated by using carbodiimide chemistry to attach ω-amino-α-carboxyl-poly(ethylene glycol) (PEG). The peptide was covalently coupled to the free terminus of the PEG, again using carbodiimide coupling. Surface contact angles for distilled water decreased from 101° initially to 61° following oxidation and 45° following PEGylation. Film surface chemistry showed the expected changes during the modifications: dye adsorption assays indicated changes in the number of acidic and basic groups and X-ray photoemission spectroscopy showed increasing oxygen and nitrogen levels. Antimicrobial activity was demonstrated in broth cultures against *E.coli*: growth was reduced by atleast 3 log cycles compared to controls. © 2008 Wiley Periodicals, Inc. J Appl Polym Sci 110: 2665–2670, 2008

Key words: peptides; poly(ethylene); surfaces; modification; antimicrobial

INTRODUCTION

Polymers are the material of choice for many applications where microbial contamination and growth is of concern. It follows that polymers with antimicrobial activity have a broad range of potential applications, including biomedical devices (e.g., surgical implants, prostheses, surgical tools); food processing equipment (food preparation and cutting surfaces, processing plant structures, polymeric gaskets and seals); and food or pharmaceutical packaging. In packaging, the antimicrobial can function either to extend product shelf life or to reduce the microbial load of the packaging prior to use. In many of these applications it is undesirable to have an antimicrobial that is released into the surrounding system. This is especially true for many biomedical devices, where systemic dispersal of the antimicrobial and/or its resulting dilution could be problematic, and it can also be true for food packaging and food processing applications. The goal of this research was to create a nonmigratory antimicrobial polymer film by covalently immobilizing an antimicrobial peptide in an active state on poly(ethylene) (PE) film.

E14LKK is a synthetic antimicrobial peptide, derived from magainin, with an amphoteric, alpha-helical structure.[1–3] It shows a broad range of antimicrobial activity against gram-positive and gram-negative bacteria, as well as yeasts and moulds.[2–5] The barrel-stave mechanism proposed by Ojcius and Young[6] is the presumed mechanism of antimicrobial activity. Minimal haemolytic activity has been demonstrated against human erythrocytes for a series of similarly sized, closely related peptides, indicating a low-likelihood of human toxicity.[7] Antimicrobial activity has been previously demonstrated with E14LKK immobilized on peptide-synthesis resins[2,4,5]; however, these resins are not amenable to further processing. In this work, we have attached E14LKK onto a potentially useful substrate, PE film, and demonstrated the antimicrobial activity of the modified film.

PE, although readily available and low cost, is inert and not inherently suited for peptide attachment. Surface oxidation has been used to create functional groups suitable for further chemical reaction.[8,9] Coupling a peptide directly to the PE, however, could potentially disrupt the peptide structure because of the hydrophobic nature of the PE, and reduce or destroy the peptide's activity. To prevent this, a hydrophilic spacer molecule, poly(ethylene glycol) (PEG), was first attached to the PE and the peptide then attached to the free terminus of the PEG. PEG has previously been successfully used for the modification of various peptides and proteins.[10] The PEG spacer additionally provides mobility to the tethered peptides, which may be required for

Correspondence to: M. D. Steven (matthew.steven@rdko.nestle.com).

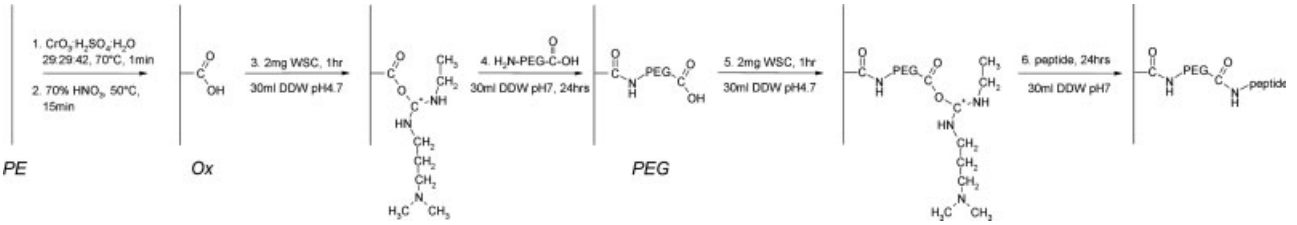

Figure 1 Film modification process. PE, Ox, and PEG designate stages of the reaction scheme. Intermediate structure based on Nakajima and Ikada.[15]

activity, given the proposed barrel-stave mechanism. Carbodiimide coupling was used to form amide bonds between an amine terminus on the PEG and carboxylic acids on the oxidized PE surface. It was additionally used to form bonds between a free carboxylic acid terminus on the tethered PEG and amines on the antimicrobial peptide. Carbodiimide chemistry has been successfully used in coupling PEG to other polymers and peptides in the past, as well as in peptide synthesis.[11–13] A range of alternative processes could be applied for each reaction step; a brief review of these possibilities, focussed on this specific application, has been published separately.[14]

METHODOLOGY

Chromium trioxide (99.75%, Mallinckrodt, St Louis, MO); sulphuric and nitric acids (AR grade, Mallinckrodt, Paris, KY); dichloromethane (DCM; AR grade, Fischer Scientific, USA); 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide and trifluoroacetic acid (TFA) (Sigma Chemicals, St Louis, MO); diamino-poly(ethylene glycol), 3400 MW (PEG-NH) and ω-amino-α-carboxyl-poly(ethylene glycol), 3400 MW (NH_2-PEG-COOH) (Shearwater Polymers, Hunstville, AL); toluidine blue O (Fisher Chemicals, Fairlawn, NJ); orange II sodium salt (Sigma-Aldrich, St Louis, MO); and bromophenol blue (BioRad, Hercules, CA) were used as purchased.

Antimicrobial peptide E14LKK, (LKKLLKLLKKLLKL; L = leucine, K = lysine), was synthesized at the Sheldon Biotechnology Center (McGill University, Montreal, Quebec). The peptide was received both as a free peptide and with side chain (lysine) amines protected with the *tert*-butyloxycarbonyl (Boc). Deprotection of the protected peptide was carried out post-coupling.

Additive-free blown low-density poly(ethylene) (LDPE) film was donated by the Dow Chemical Company (Midland, MI) and had a mean thickness (n = 10) of 100.6 μm. Films were cut into 25 mm × 50 mm pieces using a precision strip cutter (Testing Machines Incorporated, Amityville, NY), cleaned by refluxing 12–24 h in DCM and vacuum drying overnight at 60°C, ~ 27 kPa, abs., then stored in clean glass containers at ambient temperature until further treatment.

Film modification

Film modification is shown schematically in Figure 1. Films were immersed in chromic acid solution (CrO : HO : HSO = 29 : 42 : 29 by weight) at 70°C ± 2°C for 1 min, with agitation, washed thrice in distilled water, then transferred to 70% nitric acid at 50°C for 15 min and washed thrice again in distilled water.[8,9] Surface oxidized films were generally analyzed or further treated immediately. If storage was required, films were stored in distilled water under refrigeration to minimize rearrangement of the film surface and loss of surface functionality.[8] Each piece of film treated was prepared individually.

PEG attachment with carbodiimide coupling

Oxidized poly(ethylene) films were grafted with NH_2-PEG-NH_2 or NH_2-PEG-COOH using 1-ethyl-3-(3-aminopropyl)-carbodiimide (Water Soluble Carbodiimide, WSC) coupling. Three oxidized films were immersed in 30 mL of 0.35 m*M* WSC in degassed distilled water (DDW, adjusted to pH 4.5 with HCl/NaOH) at room temperature and incubated for 1 h with agitation.[12,16–18] After incubation, the films were washed thrice in distilled water and placed in 30 mL of 0.1 m*M* amino-functional PEG in DDW adjusted to pH 7, incubated for 24 h at 4°C with agitation, then removed and gently rinsed thrice in distilled water. Adsorbed PEG was removed by washing films in 40 mL of 10% aqueous ethanol (per 3 films) for 1 h with agitation. Films were rinsed thrice in distilled water, then stored under moist conditions at 4°C until further analysis or modification. Films grafted with NH_2-PEG-NH_2 were used for analyses to confirm the grafting reaction; Films grafted with NH_2-PEG-COOH were used for peptide attachment (as shown in Fig. 1).

E14LKK attachment to PE-PEG-COOH and PE-COOH

PEGylated films were treated in 0.35 m*M* WSC solution (pH 4.5, 3films + 30 mL solution) for 1 h at

room temperature with agitation, then in DDW (pH 7, 3 films + 30 mL DDW) containing the peptide. For E14LKK with unprotected side chain amines (unE14LKK), 5 mg of peptide was added to the coupling solution; for E14LKK with Boc protected side chains (proE14LKK) 5–10 mg of peptide, previously suspended in 1 mL distilled water, was added to the coupling solution. The film/peptide solutions were incubated at 4°C for 24 h with agitation, then rinsed thrice in distilled water. Films were stored at 4°C until further testing.

Deprotection of immobilized peptide E14LKK

Films with immobilized, protected peptide were immersed in 1 : 1 TFA : DCM for 30 min with agitation,[19,20] rinsed three times in distilled water, 0.1*N* NaOH, and again three times in distilled water. Deprotected films (deE14LKK) were assayed for antimicrobial activity. Preliminary tests indicated that the peptide did not show antimicrobial activity prior to deprotection (data not shown).

Surface analysis

Contact angle measurement

Films (5 mm × 50 mm) were attached to glass microscope slides with double sided tape at each end. Care was taken to only touch the ends of a film when mounting and to attach it smooth and flat; skin oils, surface buckling, and canting are all problematic for contact angle measurement.[8] A 0.5-μL drop of fluid was delivered to the film surface using a 2.5-μL hand-held syringe (Hamilton, no. 87942) with a flat-tipped, Teflon-coated needle (Hamilton, no. 80471). The drop was viewed from the side under magnification and the relative height and width of the drop were measured. The contact angle was calculated by:[21,22]

$$\tan\frac{\theta}{2} = \frac{h}{x} \quad (1)$$

where θ is the contact angle, h is the height, and x the half-width of the drop. Drops were measured within 30 s of being applied to the film. Between five and seven drops were measured for each film sample, then averaged to determine the contact angle for that sample. The results from at least three film samples were averaged to determine the contact angle for a treatment.

Dye adsorption assays

Dye absorption was used as a semi-quantitative indicator of the concentration of acid and basic functions present on the surface of the films.[23–25] To measure acid functions, film samples were soaked in 0.1 mg/mL aqueous Toluidine Blue O solution (adjusted to pH 11 with NaOH) at room temperature for 6 h, removed, rinsed in distilled water, then in 0.1 *M* NaOH and again in distilled water, then dried overnight hanging in a desiccator. The dye was desorbed by sonicating each film twice for 20 min in separate 5 mL aliquots of 1 : 1 hexane : TFA. The aliquots were combined and the absorbance measured at 677 nm. Beer's Law was used to determine dye concentration compared to standards prepared in TFA/hexane. The surface concentration of the acid groups was calculated based on the sample area, dye concentration, and volume of the desorbing solution.

To measure basic functions, films were soaked in 1.0 mg/mL aqueous Acid Orange 7 (Orange II Sodium Salt) solution (adjusted to pH 3 with HCl) at room temperature for 6 h. They were removed and rinsed in distilled water, then in 0.1*M* HCl and again in distilled water. The dye was desorbed by sonicating the film in 5 mL of 6 *N* NaOH for 20 min. The absorbance of the extraction solution was measured at 485 nm and the dye concentration calculated against standard solutions.

X-ray photoemission spectroscopy

X-ray photoemission spectroscopy (XPS) analysis was conducted using a Vacuum Generators ESCALAB Mk II (VG Scientific, West Sussex, England) with a Mg anode (1253.6 eV) operated at 15 kV and 200 W.

Antimicrobial efficacy

Films were dipped in 95% ethanol, then dried over low heat until visibly dry and placed in 10 mL of trypticase soy broth to which 0.1 mL of a 10^{-4} dilution of an overnight culture ($\sim 10^8$ cfu/mL) of *Escherichia coli* ATCC 25922 had previously been added. Sample tubes were shaken to fully immerse the films and then incubated at 37°C with gentle agitation (90 RPM) for 24 h. After incubation, serial dilutions were prepared from the test cultures ($n = 3$) and enumerated using duplicate pour plates for 10^{-5}, 10^{-6}, and 10^{-7} dilutions[26]; plates were incubated 24 h at 37°C prior to counting. Controls consisted of tubes without any film and with neither film nor inoculum, in addition to a range of control films.

Statistical analysis

All data were analyzed with ANOVAs using Minitab (v13.1 or v14.0, Minitab, State College, PA). Significant differences were determined using Tukey pairwise comparisons at the 95% overall confidence level ($\alpha = 0.05$).

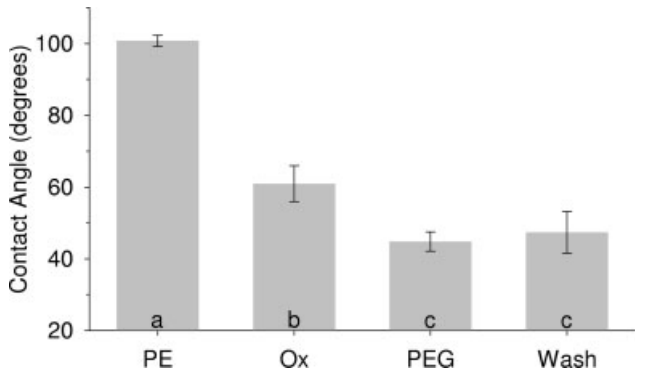

Figure 2 Water contact angles of modified LDPE ($n \geq 6$). Letters indicate significant differences.

RESULTS AND DISCUSSION

The reaction scheme used to attach E14LKK combines several previously published chemistries. These chemistries have been explored for PE surface functionalisation and peptide-polymer conjugation but have not previously been combined for peptide attachment to PE films with a PEG spacer. Carbodiimide coupling has previously been utilized to link peptides to other polymers and inorganic materials.[12,27]

Oxidation of the PE surface caused a significant decrease in contact angle (Fig. 2) from 101° to 61° for LDPE; these contact angles agree with published values.[8,28] Oxidized films also strongly adhered to glass if dried on a glass surface, while unoxidized films did not show this behavior; a similar effect was observed by Kato et al.[29] Oxidation resulted in a slight weight loss (~ 0.6 mg/25 mm × 50 mm film) of the films, probably due to surface etching, a known effect of the procedure, however, there was no measurable change in film thickness.

PEGylated films exhibited further increased surface hydrophilicity (contact angle decreased to 45°) as was expected, given the water solubility of PEG. The contact angles measured for PEGylated films were similar to those previously reported for other PEGylated polymers.[18,30–32]

Holmes-Farley et al.[8] and Sarkar et al.[32] measured the film contact angles of solutions with various pHs to probe the acidic nature of the polymer surface. At pHs corresponding to p*K*a values of acid/base groups on the surface, distinct changes were observed in the contact angle. The observed transitions were abrupt for unbuffered solutions, but broad for buffered solutions. Contact angles for buffered solutions with varying pHs were also measured here (Fig. 3). For oxidized films, the contact angle decreases as the pH increases, while no change in contact angle with pH is evident for the unmodified films. The decrease in contact angle results from increasing ionization of surface carboxylic acid groups with increasing pH; an ionized surface is more hydrophilic than a nonionized surface so has a lower contact angle. This provides evidence for the presence of acid groups on the oxidized surface.

A small decrease in contact angle with increasing pH was also observed for the PEGylated films, suggesting the presence of unreacted acid groups on the surface of the films, and indicating that the reaction does not continue to completion. The reaction was conducted with at least a 10-fold excess of PEG, so availability of reactive PEG molecules was not limiting. However, limited access of the PEG chains to reactive sites on the PE surface, due to steric hindrance, may have been an issue; a near-identical trend of contact angle with pH was observed for the washed PEGylated films.

Dye adsorption assays were used to further investigate the surface chemistry of the films (Table I) and indicated a significant increase in surface acid groups after oxidation. Others[8,9] have previously estimated a surface carboxylic acid concentration of 2.7 nmol/cm after this oxidation: the results here were of the same order of magnitude. The differences may be explained by the different methods used to determine surface acid concentrations and by incomplete dye extraction from the oxidized films: some color (and therefore dye) remained in the films even after an intense extraction procedure. For the PEGylated films, the dye absorbance assay further suggested the presence of unreacted acid groups on the surface. Unlike the oxidized films, however, a significant number of basic groups are also present on the PEGylated films, attributable to the free terminal amine functions of the PEG chains.

After washing the PEGylated film, the dye adsorption assays demonstrated the expected reduction in basic groups on the film surface. There still appear to be more basic groups present on the washed PEGylated film than on the unmodified film, although this difference is not statistically significant. The number

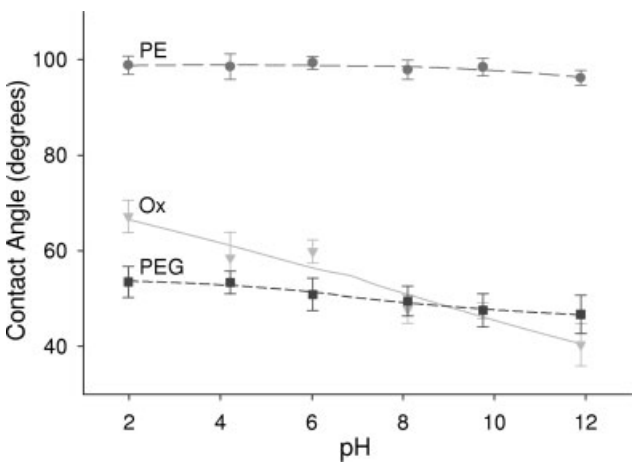

Figure 3 Effect of water droplet pH on contact angle.

TABLE I
Dye Absorbance Assays: Estimates of Surface Acid and Base Concentrations for LDPE ($n \geq 3$)

Sample	Normalized Toluidine Blue O Absorbance (A_{677} mL/cm^2)	Acid Groups (nmol/cm^2)	Normalized Acid Orange 7 Absorbance (A_{485} mL/cm^2)	Basic Groups (nmol/cm^2)
PE	0.009[a]	0	0.003[a]	0
Ox	0.066[b]	1.8	0.002[a]	0.1
PEG	0.046[c]	1.2	0.018[b]	1.3
Washed PEG	–	–	0.008[a]	0.4

PEG data is for films treated with diamino-PEG. Letters denote significant differences within a column.
– indicates not determined.

of basic groups present is at the limit of detection for the Acid Orange 7 assay, which is the probable cause of the lack of statistical significance; contact angle assays clearly indicate the presence of PEG chains on the surface of washed PEGylated films.

Dye adsorption assays conducted on films from preliminary experiments involving lactase immobilization showed a distinct increase in both acidic and basic groups compared to the unmodified and oxidized films (data not shown). The lactase was attached using amino-carboxy-PEG, so the basic groups found were not due to the presence of free terminal amines from diamino-PEG, as is the case for the PEGylated films shown in Table I. They are instead assignable to the attached lactase. Lactase attachment was also confirmed by XPS analysis (data not shown).

XPS analyses (Table II) were used to measure changes in the elemental composition of the film surfaces. As was expected, oxidation increased the oxygen content of the film surfaces. Holmes-Farley et al.[8] indicated that the oxygen functions introduced by the oxidation procedure consisted predominantly of ketones (40%) and carboxylic acids (60%). PEGylation further increased the surface oxygen content, due to oxygen in the PEG backbone, and also introduced nitrogen, from the terminal amine functions, onto the surfaces.

These data indicate that the reactions proceed as designed, first creating acid functions on the surface of the otherwise inert LDPE film, then covalently attaching PEG chains to these acid functions. The last step was the attachment of the peptide, E14LKK, to the free carboxylic acid termini of the PEG chains. The presence of the peptide was verified in antimicrobial assays (Table III).

Significant antimicrobial activity was found for films to which side-chain protected E14LKK was first immobilized and then deprotected (deE14LKK). The antimicrobial assay was conducted in growth medium to provide a comparable situation to the growth of bacteria in low-viscosity liquid foods or similar biological materials. The initial inoculum was $\sim$ 100 cfu/mL and the samples were incubated for 24 h at 37°C prior to counting. Control samples showed a 6–7 log increase in bacterial counts compared to an estimated 4 log increase in cultures exposed to film deE14LKK. This indicates a significant retardation of growth attributable to the E14LKK-modified film, even under optimal growth conditions. The count of the deE14LKK samples is termed ''estimated'' as the plates from the lowest dilution plated contained less than 30 colonies; generally a count of 30 or more colonies is required for definitive results. Hence, the actual count for E14LKK may actually be less than represented in Table III, suggesting that an even greater inhibition of growth may have occurred.

TABLE II
Elemental Composition of LDPE Film Surfaces From XPS Analyses

Sample	Carbon (%)	Oxygen (%)	Nitrogen (%)
PE	93.3	6.7	nd
Ox	86.7	13.3	nd
PEG	77.9	19.4	2.6

The base LDPE film was heat pressed in our laboratory. PEG data are for films treated with diamino-PEG. nd, not detected.

TABLE III
Antimicrobial Activity of Modified Films

Film	Microbial Count log (cfu/mL)
Control	9.1 (0.1)[a]
PE	8.8 (0.1)[a]
Ox	8.6 (0.2)[a]
PEG	9.0 (0.1)[a]
Wash	8.1 (0.2)[a]
unE14LKK	8.1 (0.2)[a]
deE14LKK	5.9 (0.3)[b,1]

Mean values are followed (in brackets) by standard deviations (n = 3). Significant differences are indicated by differing letters.
[1] Estimated count, all plates had less than 30 colonies.

In addition to the work with LDPE, modification of high-density poly(ethylene) (HDPE) was also investigated. The modification scheme was also successful for HDPE, although it was only conducted as far as PEGylation and washing (data not shown). No peptide immobilization or bioactivity analyses were conducted with HDPE because of limited peptide supply. Equivalent results would be expected, however, to those observed for LDPE.

To summarize, the combined results from contact angle, dye adsorption, and XPS analyses indicate that the desired chemical changes occur at the surface of the PE film. After attaching and then deprotecting antimicrobial peptide E14LKK, an antimicrobial LDPE film was successfully obtained. Results from studies on the physical, optical, and mechanical properties of the modified films are forthcoming.

Our thanks to Dr Michael Kelley and colleagues at Jefferson Laboratory (Newport News, VA) for conducting the XPS analyses and aiding in their interpretation.

References

1. Zasloff, M. Proc Natl Acad Sci USA 1987, 84, 5449.
2. Haynie, S. L.; Crum, G. A.; Doele, B. A. Antimicrob Agents Chemother 1995, 39, 301.
3. Haynie, S. L. U.S. Pat. 5,847,047 (1998).
4. Appendini, P. Ph.D. Thesis, Cornell University, Ithaca, NY, 1999.
5. Appendini, P.; Hotchkiss, J. H. J Appl Polym Sci 2001, 81, 609.
6. Ojcius, D. M.; Young, J. D.-E. Trends Biochem Sci 1991, 16, 225.
7. Blondelle, S. E.; Houghten, R. A. Biochemistry 1992, 31, 12688.
8. Holmes-Farley, S. R.; Reamey, R. H.; Mccarthy, T. J.; Deutch, J.; Whitesides, G. M. Langmuir 1985, 1, 725.
9. Rasmussen, J. R.; Stedronsky, E. R.; Whitesides, G. M. J Am Chem Soc 1977, 99, 4736.
10. Veronese, F. M. Biomaterials 2001, 22, 405.
11. Bodanszky, M. Peptide Chemistry: A Practical Textbook; Springer-Verlag: Berlin, 1993.
12. Lin, J. C.; Tseng, S. M. J Mater Sci: Mater Med 2001, 12, 827.
13. Valuev, I. L.; Chupov, V. V.; Valuev, L. I. Biomaterials 1998, 19, 41.
14. Steven, M. D.; Hotchkiss, J. H. In Novel Food Packaging Techniques; Ahvenainen, R., Ed.; Woodhead Publishing: Cambridge, England, 2003; pp 71–102.
15. Nakajima, N.; Ikada, Y. Bioconjugate Chem 1995, 6, 123.
16. Anon. Sigma-Aldrich Website http://www.sigmaaldrich.com/ Sigma-Aldrich Co.: St. Louis, MO, 2002.
17. Anon. Current Protocols in Protein Science; Chapter 18: Preparation and Handling of Peptides, http://www.mrw2.interscience.wiley.com/cponline/John Wiley & Sons, Inc., 2002.
18. Bae, J. S.; Seo, E. J.; Kang, I. K. Biomaterials 1999, 20, 529.
19. CalBiochem-NovaBiochem Corporation. NovaBiochem 1999 Catalog and Peptide Synthesis Handbook; San Diego, CA, 1999.
20. Pennington, M. W. In Peptide Synthesis Protocols; Pennington, M. W., Dunn, B. M., Eds.; Humana Press: Totowa, NJ, 1994; p 41–62.
21. Bartell, F. E.; Zuidema, H. H. J Am Chem Soc 1936, 58, 1449.
22. Mack, G. L. J Phys Chem 1935, 40, 159.
23. Hu, S. G.; Jou, C. H.; Yang, M. C. J Appl Polym Sci 2002, 86, 2977.
24. Kato, K.; Ikada, Y. Biotechnol Bioeng 1996, 51, 581.
25. Sano, S.; Kato, K.; Ikada, Y. Biomaterials 1993, 14, 817.
26. Speck, M. L. Compendium of Methods for the Microbiological Examination of Foods; American Public Health Association, Inc: Washington, DC, 1984.
27. Plummer, S. T.; Bohn, P. W. Langmuir 2002, 18, 4142.
28. Schonherr, H.; Vancso, G. J. J Polym Sci Part B: Polym Phys 1998, 36, 2483.
29. Kato, K.; Uchida, E.; Kang, E.-T.; Uyama, Y.; Ikada, Y. Prog Polym Sci 2003, 28, 209.
30. Gombotz, W. R.; Guanghui, W.; Hoffman, A. S. J Appl Polym Sci 1989, 37, 91.
31. Kiss, E.; Samu, J.; Toth, A.; Bertoti, I. Langmuir 1996, 12, 1651.
32. Sarkar, N.; Bhattacharjee, S.; Sivaram, S. Langmuir 1997, 13, 4142.