Poly(lactic acid)/Soluble Eggshell Membrane Protein Blend Films: Preparation and Characterization

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Received 31 May 2009; accepted 30 January 2010
DOI 10.1002/app.32181
Published online 1 April 2010 in Wiley InterScience (www.interscience.wiley.com).

ABSTRACT: Poly(lactic acid) (PLA), one of the most commonly used synthetic biodegradable polymers having good mechanical properties, is blended with soluble eggshell membrane protein (SEP) by solution casting from hexafluoroisopropanol to improve the mechanical properties of SEP. When PLA/SEP proportion is 9 : 1, the cast film has much better mechanical properties than neat SEP film and significantly improved biocompatibility compared to neat PLA film, as demonstrated by tensile tests and cell culture of NIH3T3. The PLA/SEP blend films were further characterized by field emission gun scanning electron microscopy, attenuated total reflectance Fourier transform infrared spectroscopy, differential scanning calorimetry, X-ray diffraction, and contact angle measurements. © 2010 Wiley Periodicals, Inc. J Appl Polym Sci 117: 1955–1959, 2010

Key words: soluble eggshell membrane protein; poly(lactic acid); blending; mechanical properties; biocompatibility

INTRODUCTION

Soluble eggshell membrane protein (SEP),[1,2] isolated from natural eggshell membrane (ESM), which is composed mainly of collagen, has shown good biocompatibility and biodegradability[1–3] and thus is a potential proteinous biomaterial. However, its mechanical properties are poor. The cast film is very brittle. Blending[4,5] with other polymers having good mechanical properties is a convenient way to obtain biomaterials suitable for practical applications such as tissue engineering scaffold and biomedical materials, and can be used to improve mechanical properties of SEP-based cast film. Synthetic polymer poly(vinyl alcohol) (PVA) and biopolymer chitosan were previously blended with SEP by solution casting.[6,7] Both PVA/SEP and chitosan/SEP blend films have considerably improved mechanical properties, while the good biocompatibility of SEP is largely maintained. However, both PVA/SEP and chitosan/SEP films were cast from aqueous solutions. Their anti-water properties are poor. Both types of films swell to some extent in water and in cell culture medium. Also, the biodegradability of PVA is questionable.

Blending with water-insoluble and biodegradable polymers could resolve the aforementioned drawbacks. Similar to collagen, SEP is also soluble in organic solvent hexafluoroisopropanol (HFIP),[8,9] a powerful solvent of many common polymers such as poly(lactic acid) (PLA), poly(propylene carbonate) (PPC), and polyamides. This solubility should allow processing of this type of blending through solution casting. PLA[10–12] is one of the most commonly used synthetic polymers in medical field. It has good mechanical properties, biodegradability, and water-insolubility. It could be an ideal candidate for blending with SEP. SEP was previously immobilized on PLA membrane surface by a physical entrapment strategy involving multisteps: swelling, contraction, and cross-linking.[13]

In this article, PLA is blended with SEP by solution casting from HFIP. The goal is to provide a simple alternative method to PLA/SEP hybrid biomaterial having simultaneously good mechanical properties, biocompatibility, biodegradability, and anti-water properties. The mechanical properties are measured by tensile tests, and the biocompatibility is evaluated by cell culture of NIH 3T3 (Mouse embryonic fibroblast cell line). The phase morphology of PLA/SEP blends, interaction between SEP and PLA, crystallization behavior of PLA, and surface wettability of PLA/SEP films are also investigated.

EXPERIMENTAL

Materials

SEP was prepared by dissolving raw ESM powder in aqueous 3-mercaptopropionic acid and acetic acid

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Contract grant sponsor: National Natural Science Foundation of China; contract grant number: 50773035.

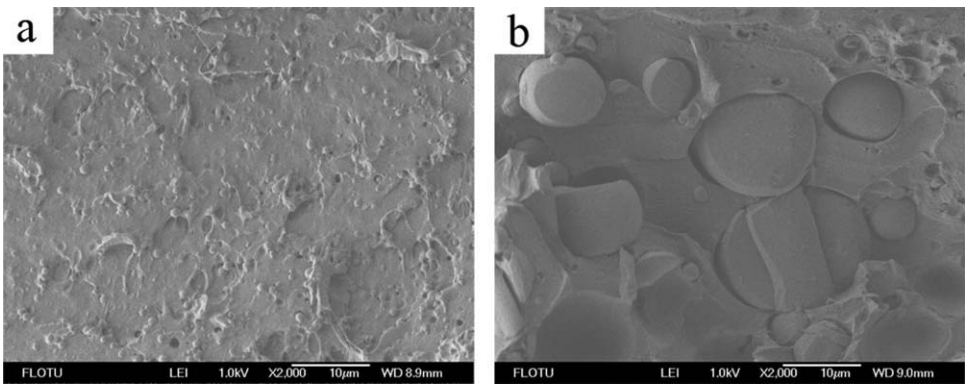

Figure 1 FESEM photomicrographs of cross sections of PLA/SEP blend films (a) 9 : 1 and (b) 7 : 3.

followed by neutralizing to pH 5 according to our previous report.[1,2] PLA (100 kDa) used in this work was racemic poly(D,L-lactic acid) and provided by Wuhan Huafu Tech Co, China. HFIP was purchased from Shanghai Humei Co, China.

Preparation of PLA/SEP blend films

The PLA/SEP blend solutions were prepared by dissolving PLA in a solution of SEP in HFIP. The total concentration of PLA and SEP was fixed at 3 wt %. The PLA/SEP blend films were obtained by casting the blend solutions onto glass plates with a diameter of 10 cm and allowing the solvent to evaporate slowly at room temperature. The volume range of the solution for casting each film was 20–25 mL depending on PLA/SEP ratio. Then the films were peeled off and dried in a vacuum oven at 30°C to remove the remaining HFIP. The thickness of the films was 0.015–0.020 mm.

Measurements

The phase morphology of the cross sections of the blend films was examined with a JSM-7401 Field Emission Gun Scanning Electron Microscope (FESEM) after being fractured in liquid nitrogen. Attenuated total reflectance Fourier transform infrared (ATR-FTIR) spectra were obtained with a Fourier transform spectrophotometer (Nicolet, MAGNA-IR 560) coupled with an ATR accessory (split-pea) by averaging 32 scans at a resolution of 4 cm^{-1} in the range 4000–650 cm^{-1}. Differential Scanning Calorimetry (DSC) was performed on a TA DSC-2910 differential scanning calorimeter, the samples were heated from 30°C to 190°C at the rate of 10°C/min in flowing N_2. The X-Ray Diffraction (XRD) measurements were carried out at room temperature on a Bruker D8-Advance diffractometer (CuKα) connected to a computer. The diffraction scans were collected at 1.5–40°.

Tensile strength and elongation-at-break were determined by a TS-2000 testing machine, and a cross-head speed of 10 mm/min was employed. Samples were made by cutting the films into rectangles of 50 mm × 10 mm. Each sample's thickness was measured before testing.

The water contact angles of the blend films were measured at room temperature using the COA2000 type contact angle analyzer (Dataphysic, Germany). A water droplet of 4 μL was put on the surface of a small sample. The shape of the droplet was captured and image analysis software was used to determine the contact angle. Five independent determinations at different sites were averaged.

Cell culture

NIH3T3 cells were cultured in Dulbecco's Minimum Essential Medium (DMEM, Gibco) with 10% fetal bovine serum (FBS, Gibco) in a humidified incubator in an atmosphere of 5% CO_2 at 37°C. The medium was changed every 2 days. The cell monolayer was washed twice by Phosphate Buffered Saline (PBS) and incubated with trypsin-EDTA solution (0.25% trypsin, 1 m*M* EDTA; Gibco) for 3 min at 37°C to detach the cells. The effect of trypsin was then inhibited by adding the complete medium at room temperature and reseeding and growing the cells in new culture flasks.

Cell proliferation

SEP/PLA blend films containing different amounts of SEP were cut into small disks to locate the disks into 24-well tissue culture plates. All samples were sterilized by ultraviolet ray for 25 min, and soaked in DMEM for 24 h to remove the remaining impurities. Then the DMEM in 24-well tissue culture plates was removed by a pipette, NIH3T3 cells were seeded with a density of ~10,000 cells/cm^2. The cells were allowed to attach to the films undisturbed in a

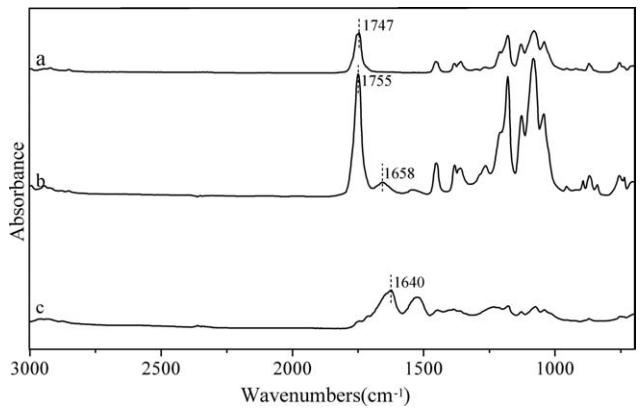

Figure 2 ATR-FTIR spectra of PLA/SEP blends (a) 1 : 0, (b) 9 : 1, and (c) 0 : 1.

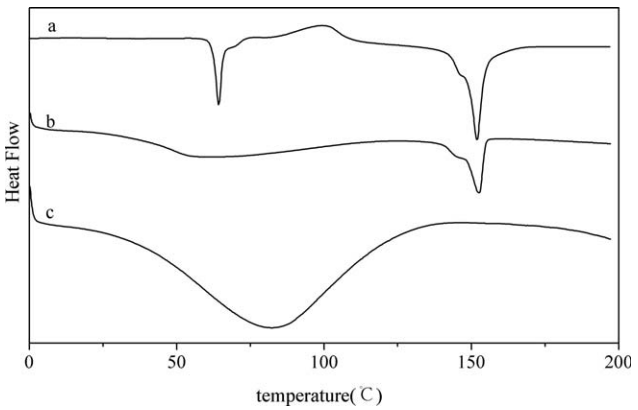

Figure 3 DSC curves of PLA/SEP blend films (a) 1 : 0, (b) 9 : 1, and (c) 7 : 3.

humidified incubator (37°C and 5% CO_2) for 1 and 3 days.

A 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) assay was used to examine the cell viability. After each period of culturing time in 24-well plates, MTT solution (5 mg/mL, 100 μL, Sigma) was added to each well. After 4 h incubation at 37°C, dimethyl sulfoxide (DMSO) (500 μL) was added to dissolve the formazan crystals and transferred to a 96-well plate. The optical density of the formazan solution was measured on an ELISA plate reader (Spectra Max 190, Molecular Devices) at 570 nm.[14]

Cell morphology observation

NIH3T3 cells were cultured on SEP/PLA blend films for 3 days and then fixed with 3% glutaraldehyde in PBS for 3 h at 4°C. After washing with PBS, the samples were hydrated sequentially in 50, 60, 70, 80, 90, and 100% ethanol for 10 min each. The fixed samples were sputter-coated with gold and observed under FESEM (JSM 7401).

RESULTS AND DISCUSSION

Phase morphology

Homogeneous PLA/SEP solutions were obtained when PLA was added to SEP solution in HFIP in different PLA/SEP proportions (9 : 1, 7 : 3, and 1 : 1). Evaporation of HFIP gave films with macroscopic phase separation when PLA/SEP proportion is 1 : 1, and thus this composition was not investigated further. Homogeneous films without macroscopic phase separation were obtained with PLA/SEP 9 : 1 and 7 : 3 blends, indicating partial compatibility of the two components (PLA and SEP) at low SEP contents.

The phase morphology of PLA/SEP 9 : 1 and 7 : 3 films were investigated by FESEM and the micrographs of cryo-fractured cross sections are shown in Figure 1. Micro-size phase separation was observed. When SEP content is 10%, the average particle size is 1.1 μm. With more incorporation of SEP (30%), the average particle size increases to 8.1 μm. The average particle size is about one order of magnitude larger than that in PVA/SEP blend films (0.1 ~ 0.4 μm),[7] suggesting that SEP is less compatible with PLA than with PVA.

Interaction between SEP and PLA

Interaction between the two components is very important for polymer blends, because it affects phase morphology and mechanical properties of the blends. In this work, ATR-FTIR was used to investigate possible interaction between PLA and SEP, and the spectra of PLA/SEP 9 : 1, neat SEP and neat PLA films are shown in Figure 2. With incorporation

TABLE I
Properties of PLA/SEP Blend Films

PLA/SEP	Melting temperature (°C)	Enthalpy of fusion (J/g)	Tensile strength (MPa)	Elongation to break (%)	Contact angle (°)
1 : 0	151.9	24.2	43.1	26	76.2 ± 1.4
9 : 1	152.6	13.7	32.9	18	49.4 ± 2.0
7 : 3	–	–	22.4	6	35.8 ± 4.7
0 : 1	–	–	–	–	53.4 ± 2.5

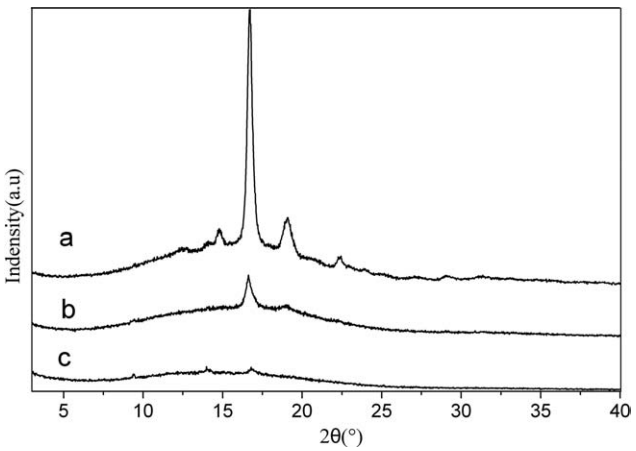

Figure 4 XRD patterns of PLA/SEP blend films (a) 1 : 0, (b) 9 : 1, and (c) 7 : 3.

of PLA, the amide I band of SEP is shifted from 1640 to 1658 cm^{-1}, and the carbonyl band of PLA is shifted from 1747 to 1755 cm^{-1}. This suggests that intermolecular interaction exists between PLA and SEP. Presumably, the carbonyl groups of PLA interact with the amide or carboxyl groups of SEP through hydrogen bonding, and this type of interaction replaces some inter- or intramolecular hydrogen bonding among SEP molecules.

Crystallization behavior of PLA

Crystallization of materials can affect its mechanical properties. In this work, DSC and XRD were used to study the crystallization behavior of the blend films. DSC curves of neat PLA and PLA/SEP 9 : 1 and 7 : 3 films are shown in Figure 3. The melting temperature and enthalpy of fusion are listed in Table I. The glass transition temperature (T_g) of PLA is not observed clearly for all the tested samples due to interruption of the unknown peak at 65.5°C for neat PLA sample and the broad water peak at 50 ~90°C for blend samples containing SEP which is hydrophilic. Cold crystallization is observed for neat PLA film at 110.0°C, but not observed for the blend films. Melting of PLA is observed for neat PLA and PLA/SEP 9 : 1 films at 152°C, but not observed for PLA/SEP 7 : 3 film, indicating that the PLA/SEP 7 : 3 film is amorphous. As mentioned earlier, interaction between PLA and SEP exits. This interaction inhibits the regular arrangement of PLA molecules, leading to amorphous material when SEP content is relatively high (30%).

The XRD patterns of neat PLA and PLA/SEP 9 : 1 and 7 : 3 films are shown in Figure 4. Neat PLA film has a strong diffraction peak at $2\theta = 16.6°$ and a weak peak at $2\theta = 19°$, corresponding to the (110) and (203) planes reflections.[15] With the addition of 10% SEP, the peaks are weakened. When the content of SEP is 30%, amorphous pattern is observed. The results from DSC and XRD are basically in agreement.

Mechanical properties

Tensile tests were used to evaluate the mechanical properties of PLA/SEP blend films, and the results are given in Table I. The tensile strength and elongation to break of neat PLA film are 43.1 MPa and 26%, respectively. Neat SEP film was too brittle to measure. When SEP is blended to PLA, both tensile strength and elongation to break decrease compared to those of neat PLA film. Negative deviation from the linear mixing rule is observed due to phase separation (as revealed by FESEM) and the decrease in crystallinity of PLA (as shown by XRD and DSC). The PLA/SEP 9 : 1 film has fairly good mechanical properties. The PLA/SEP 7 : 3 film is somewhat brittle due to increased phase separation as shown by FESEM.

Wettability

Surface wettability is an important parameter for biomaterials. In general, hydrophilic surface favors cell adhesion and growth.[10] Static water contact angle measurements were used to characterize the hydrophilicity of PLA/SEP 9 : 1 and 7 : 3 blend

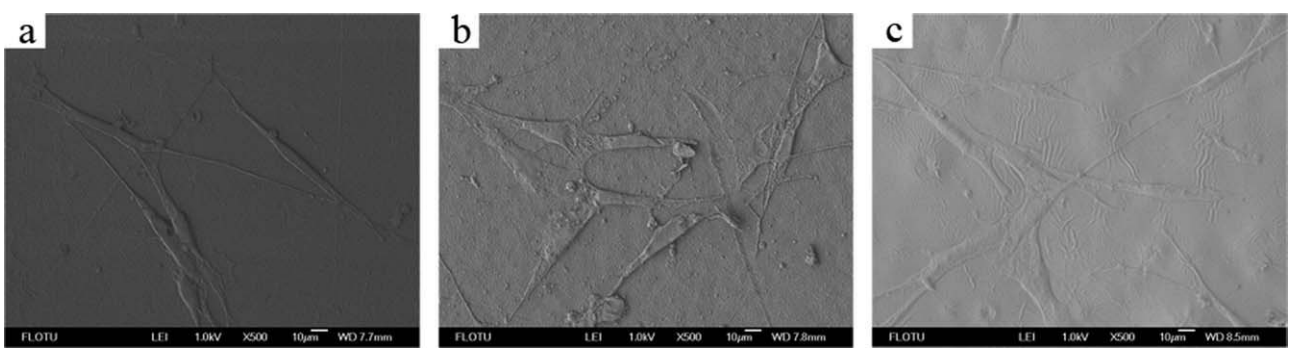

Figure 5 FESEM photomicrographs of NIH3T3 cultured on PLA/SEP blend films after 3 days culturing (a) 1 : 0, (b) 9 : 1, and (c) 7 : 3.

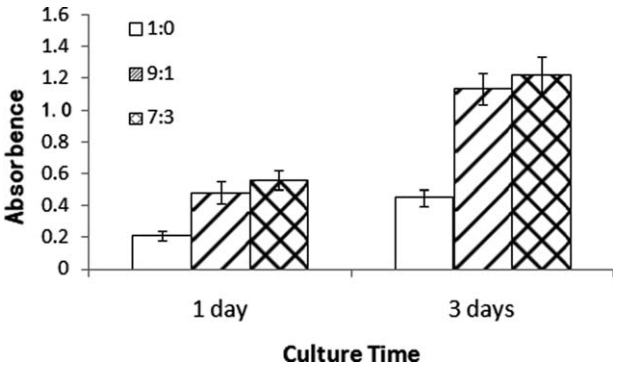

Figure 6 MTT assays of NIH3T3 seeded on PLA/SEP blend films with ratio of 1 : 0, 9 : 1, and 7 : 3. Formazan absorbance is expressed as a function of culture time. Error bars represent the standard error of the mean ($n = 5$).

films, neat PLA and SEP films. As listed in Table I, the contact angle of neat PLA film is 76.2°. After blending with SEP, the contact angle decreases to 49.4° (with 10% SEP) and 35.8° (with 30% SEP), which are even lower than that of neat SEP film (53.4°). The improved surface hydrophilicity is due to the presence of hydrophilic component SEP and presumably also the increase in surface roughness which could be caused by phase separation.

Biocompatibility

The biocompatibility of the PLA/SEP blend films (9 : 1 and 7 : 3) was evaluated by cell culture, and compared with that of neat PLA film. No obvious swelling and weight loss were observed for the PLA/SEP blend films after immersing in the cell culture medium at 37°C for 72 h, indicating good anti-water property. Figure 5 shows the cell morphology after three days' culture on the aforementioned three different substrates. In the cases of PLA/SEP blend films, the numbers of the cells are greater and the cells spread out more than in the case of neat PLA, indicating better cell adhesion and proliferation on the blend films. This is mainly due to the incorporation of SEP, which is known to have excellent biocompatibility.[1,2]

Figure 6 shows the cell viabilities of NIH3T3 cells cultured on all the three substrates at day 1 and 3. The cells proliferate on all the three substrates, and the cell viability of NIH3T3 cultured on the PLA/SEP blend films is always much higher (more than double) than that cultured on neat PLA. No obvious difference in cell viability was observed with the two PLA/SEP blend films having different SEP contents (10% and 30%), suggesting that incorporation of 10% SEP is enough to significantly improve the biocompatibility of PLA.

CONCLUSIONS

PLA can be used to improve the mechanical properties of SEP. PLA/SEP films containing up to 30% SEP can be prepared without macroscopic phase separation by solution casting from HFIP. Microscopic phase separation does occur, and the particle size increases with the increase in SEP content from 10% to 30%. Intermolecular interaction between PLA and SEP exists due to hydrogen bonding between the carbonyl groups of PLA and the amide or carboxyl groups of SEP. PLA is semicrystalline in PLA/SEP 9 : 1 film, while amorphous in PLA/SEP 7 : 3 film. The surface hydrophilicity of the blend films is significantly improved compared to PLA. The blend films show good anti-water property. The PLA/SEP 9 : 1 film has fairly good mechanical properties, much better than those of neat SEP film, and considerably improved biocompatibility as compared to neat PLA film.

References

1. Yi, F.; Guo, Z. X.; Zhang, L. X.; Yu, J.; Li, Q. Biomaterials 2004, 25, 4591.
2. Yi, F.; Yu, J.; Guo, Z. X.; Zhang, L. X.; Li, Q. Macromol Biosci 2003, 3, 234.
3. Yi, F.; Yu, J.; Li, Q.; Guo, Z. X. J Wuhan Univ Technol 2007, 22, 117.
4. Peschel, G.; Dahse, H. M.; Konrad, A.; Wieland, G. D.; Mueller, P. J.; Martin, D. P.; Roth, M. J Biomed Mater Res A 2008, 85, 1072.
5. Suyatma, N. E.; Copinet, A.; Tighzert, L.; Coma, V. J Polym Environ 2004, 12, 1.
6. Qi, Q. L.; Li, Q.; Lu, J. W.; Guo, Z. X.; Yu, J. Chin J Polym Sci 2009, 27, 387.
7. Yi, F.; Lu, J. W.; Guo, Z. X.; Yu, J. J Biomater Sci Polym E 2006, 17, 1015.
8. You, Y.; Lee, S. J.; Min, B.; Park, W. H. J Appl Polym Sci 2006, 99, 1214.
9. Min, B. M.; Lee, S. W.; Lim, J. N.; You, Y.; Lee, T. S.; Kang, P. H.; Park, W. H. Polymer 2004, 45, 7137.
10. Wang, S. G.; Cui, W. J.; Bei, J. Z. Anal Bioanal Chem 2005, 381, 547.
11. Gupta, B.; Revagade, N.; Hilborn, J. Prog Polym Sci 2007, 32, 455.
12. Auras, R. A.; Harte, B.; Selke, S.; Hernandez, R. J Plast Film Sheet 2003, 19, 123.
13. Lu, J.; Li, Q.; Qi, Q. L.; Guo, Z. X.; Yu, J. J Biomed Mater Res A, 2009, 91A, 701.
14. Mosmann, T. J Immunol Methods 1983, 65, 55.
15. Park, J. W.; Im, S. S. J Appl Polym Sci 2002, 86, 647.