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Improvement in physical properties and cytocompatibility of zein by incorporation of pea protein isolate

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Abstract A series of protein-based biocomposites was prepared from glycerol-plasticized zein (ZE) and pea protein isolate (PPI) using a hot press and moulding process. The effects of PPI content (W_{PPI}) on the structure and properties of ZE/PPI films were investigated. With an increase in W_{PPI} from 0 to 100%, the elongation at break of the films increased from 2.4 to 62.6%, and the water contact angle decreased from 31.8° to 5.8°. Cell toxicity and cytocompatibility of ZE/PPI films were evaluated in vitro. The cell viability of L929 cells cultured in extracts from ZE/PPI films containing 10–30% PPI was higher than that from other films and the control group. The L929 cells expanded very well on the surfaces of films containing 10–30% PPI. Incorporation of 10–30% PPI into ZE improved flexibility, surface hydrophilicity, cytocompatibility and its potential as biomaterials in zein-based composites.

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Introduction

Zein, the prolamine fraction of corn protein, is produced commercially from corn gluten meal [1]. It is known as a hydrophobic protein due to its low polar and high nonpolar amino acid contents [2]. As a result of zein's good solubility in aqueous alcohol solutions, it is easily fabricated into films using a casting method [3, 4]; into microspheres using a phase separation process [5, 6]; and into fibres using an electrospinning technique [7]. By adding plasticizers such as water, glycerol, tri(ethylene) glycol, polyethylene glycol, oleic or linoleic acids or sugars (fructose, galactose and glucose) [4, 8–10], zein's thermoplasticity allows it to be extruded and hot pressed into films, sheets or scaffolds [11].

Zein is rarely used directly for human consumption because of its negative nitrogen balance and poor solubility in water. Current and potential applications for zein-based materials are in the specialty food, biodegradable plastics, pharmaceutical and medical industries [1, 12]. Applications for zein in the medical industry will greatly increase its value. A series of previous reports showed that, due to its nontoxicity, biodegradability and good biocompatibility, zein is a promising natural polymer for the development of biomaterials for tissue engineering [13–16], drug delivery systems [5, 8, 17, 18], biocompatible coating materials for medicine or medical devices [1, 19, 20], antioxidants [21] and anticoagulants [22]. Zein films with different surface morphologies and microstructures have been prepared by dissolving zein in 70% alcohol solution, with or without ultrasonic treatment, followed by a casting and evaporation process [13]. Results from cell culture experiments showed that over 60% of both human liver cells (HL-7702) and mouse fibroblast cells (NIH3T3) attached to these zein films 3 h after seeding the cells onto

the materials. The cells grew well on the surfaces of the zein films during the culture process, which showed that zein exhibits good cytocompatibility and has potential for application as a tissue scaffold. In other work [23], it was reported that humidity and micro-morphology of the surfaces were responsible for cell behaviour and blood adsorption on the surface of the zein films. The use of zein as a matrix or scaffold for cell seeding in tissue engineering is often limited by its poor mechanical performance and the loss of biological properties during formulation or practical utilization [13, 24]. Films prepared from zein showed high tensile strength and modulus with very poor toughness and flexibility due to their brittle nature [3]. Another shortcoming of zein as a biomaterial is its high hydrophobicity, which has a negative effect on cell adhesion, immigration and proliferation on the hydrophobic surface of the zein films or on the interior of zein scaffolds. Thus, improvement of the mechanical properties and adjustment of the surface hydrophilicity/hydrophobicity of zein-based materials are two key points for developing its applications as a biomaterial.

Compared with zein and soy protein isolate (SPI), commercially available pea protein isolate (PPI) is an underutilized plant protein [25]. PPI contains two globulin-type storage proteins: legumin and vicilin. The former fraction is a hexameric 11*S* protein (350–400 kDa) containing disulphide bridges, whereas the latter is a trimeric 7*S* protein with a molecular mass of 150 kDa [26]. Currently, PPI is utilized primarily in nutritional supplements, biodegradable edible films, gels and coating materials [27–30]. In recent years, the application of SPI as a biomaterial has been widely reported [31, 32]. The protein fractions (7*S* and 11*S* globular proteins) and physical properties of PPI are very close to SPI, which hints that PPI would have the same potential as SPI in the biomedical field; however, research reports on the application of PPI as a biomaterial are absent.

Examining the physical properties of zein and PPI, it has been found that zein generally has high hydrophobicity and low flexibility, while PPI generally has poor water-resistance and high flexibility. Thus, in order to expand the applications of two underutilized plant proteins (i.e. zein and PPI) into biomaterials, we prepared a series of glycerol-plasticized zein/PPI composites without using chemical reaction. In this work, it was hypothesised that PPI would contribute to the improvement of mechanical properties and to the adjustment of the hydrophilicity/hydrophobicity of zein/PPI composites. Meanwhile, zein would contribute to an increase in the water-resistance of the PPI component and maintain the biocompatibility of the zein-based materials. The effects of the components and structure of the composites on the cytotoxicity and cytocompatibility were investigated by cell culture experiments.

Materials and methods

Materials

Zein from maize (protein content >90%) was purchased from Sigma–Aldrich Canada Ltd. (Oakville, Canada). Pea protein isolate (Propulse^{TM}, prepared from Canadian yellow peas) was supplied by Nutri-Pea Limited Canada (Portage la Prairie, Canada). According to the manufacturer's analytical reports, it contained 6% moisture, <4% ash, <3% lipids, <12% carbohydrates and 82% protein. Zein and pea protein isolate powders were vacuum-dried at 50 °C for 24 h before use. Glycerol (99.5%) and other chemicals were purchased from Sigma–Aldrich Canada Ltd. (Oakville, Canada) and used without further purification.

Preparation of zein/pea protein isolate biocomposites

Zein and glycerol were mixed (weight ratio of zein/glycerol was 30/70) and pestled in a mortar for 30 min, then extruded three times through a 1.2 mm 25% dome die using a Fuji Paudal MG55 granulator (LCI Corporation, Charlotte, NC, USA) to obtain a zein-based mixture (coded as ZE). Pea protein isolate-based mixture (weight ratio of pea protein isolate/glycerol was also 30/70) was prepared by the same process as the ZE and coded as PPI. The ZE and PPI were blended together and extruded three times to form glycerol-plasticized ZE/PPI mixtures. By changing the weight percent of PPI (W_{PPI}) to 0, 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 wt%, a series of ZE/PPI composites was obtained. The extruded composites were sealed in plastic bags and stored at 4 °C for 3 days. Three grams of the extruded material was placed into a mould, covered on both sides with polished stainless steel plates, and compression-moulded using a Carver Auto M Press (Carver Inc., Wabash, IN, USA). The mould temperature was kept at 140 °C, and the pressure was increased from 0.5 to 20 MPa within 1 min and held there for 12 min. The mould was cooled to 30 °C with a water and air cooler system. The compressed film was then released from the mould and stored in a desiccator. The series films were coded as ZE/PPI-*n*, where *n* was the percent of PPI in the ZE/PPI composites. The codes and sample compositions are listed in Table 1.

Characterization of structure and physical properties

Fourier transform infrared (FTIR) analysis

Attenuated total reflection Fourier transform infrared (ATR-FTIR) spectra of the hot press ZE/PPI films were

Table 1 Codes, compositions and cell culture results for the ZE/PPI-*n* series composite films

Sample code	W_{PPI} (%)[a]	Cell density (cell number mm^{-2})	Percentage of shuttle shaped cells (%)	Percentage of round shaped cells (%)
Control	–	1671	95.3	4.7
ZE/PPI-0	0	2307	29.8	71.2
ZE/PPI-10	10	2062	90.0	10.0
ZE/PPI-20	20	Δ	Δ	Δ
ZE/PPI-30	30	1576	88.0	12.0
ZE/PPI-40	40	Δ	Δ	Δ
ZE/PPI-50	50	1549	11.5	88.5
ZE/PPI-60	60	Δ	Δ	Δ
ZE/PPI-70	70	814	18.9	81.1
ZE/PPI-80	80	Δ	Δ	Δ
ZE/PPI-90	90	991	3.6	96.4
ZE/PPI-100	100	624	2.5	97.5

Control: glass plate

ZE: Mixture of 70% zein powder and 30% glycerol; PPI: Mixture of 70% pea protein isolate powder and 30% glycerol

[a] W_{PPI} = [Weight of PPI/(Weight of ZE + weight of PPI)] × 100%

Δ: No cell culture experiment for the corresponding sample

recorded on a Fourier transform infrared spectrometer (170SX, Nicolet Co., Madison, WI, USA). The data were collected over 16 scans with a resolution of 4 cm^{-1} in a wavelength range 4000–650 cm^{-1}.

Morphology of the films

A scanning electron microscope (SEM, S-4800, Hitachi, Ibaraki, Japan) was used to observe the morphology of the original materials and the resulting films. The dried films were frozen in liquid nitrogen and immediately fractured. The dried ZE and PPI powders and the cross-sections of the ZE/PPI-*n* films were then coated with gold and observed with an accelerating voltage of 5 kV.

Thermogravimetric (TG) and differential thermogravimetric (DTG) analyses

Thermogravimetric (TG) analysis and differential thermogravimetry (DTG) of the selected ZE/PPI-*n* films were carried out on a TA-STDQ600 (TA Instruments Inc., New Castle, DE, USA). The thermograms were acquired between 30 and 480 °C at a heating rate of 10 °C/min. Nitrogen was used as the purge gas at a flow rate of 20 mL/min.

Tensile testing

The tensile strength (σ_b), elongation at break (ε_b) and Young's modulus (E) of the films were measured on a universal testing machine (CMT6500, Shenzhen SANS Test Machine Co. Ltd., Shenzhen, China) according to ISO527-2:1993 with a tensile rate of 10 mm min^{-1}. The mean values were obtained from measurements of more than three specimens and the standard deviations were calculated. The films were kept in a desiccator with a relative humidity (RH) of 53% (controlled by saturated KNO_3 solution) for 1 week before testing.

Measurement of water contact angle

The water contact angles of the films were measured on a C201 contact angle system (Solon Tech. Inc. Ltd. Shanghai, China) with deionized water as the probe liquid. A water droplet was deposited on the sample surface, and a charged coupling device video camera and image analysis software were used to determine the contact angle value. The results are the average values of two samples.

Weight change and weight loss of films soaked in water

The ZE/PPI-*n* films were vacuum-dried at 50 °C for 24 h and subsequently cooled in a desiccator before weighing (W_0). Each film was soaked with 50 mL distilled water in a sealed glass bottle at 25 °C for t (t = 0–168 h). The surface moisture was then wiped off with a paper towel and the film weighed (W_t) again. The distilled water in the bottle was kept constant of 50 mL during the testing process. The weight change (W_{change}) of the samples after the soaking process was calculated as follows:

$$W_{change} = [(W_t - W_0)/W_0] \times 100\% \quad (1)$$

When t = 168 h, the samples were taken out of the water and vacuum-dried at 50 °C for 24 h. The dry weight (W_{dry}) of the samples was then determined. The percentage weight loss (W_{loss}) of the films soaked in water for 168 h was calculated as follows:

$$W_{loss} = \left[(W_0 - W_{dry})/W_0\right] \times 100\% \quad (2)$$

In vitro evaluation of cytotoxicity and cytocompatibility

Preparation of film extracts

Extracts from sterilized ZE/PPI-*n* films were prepared according to ISO 10993-12:2002. Pre-weighed ZE/PPI-*n* ($n = 0$, 10, 30, 50, 70, 90 or 100) films were incubated in cell culture medium (DMEM) (0.1 g film per 1 ml DMEM) at 37 °C for 24 h. The extracts were then stored at 4 °C.

Cytotoxicity evaluation by MTT

According to ISO 10993-5, a cell line of mouse lung fibroblasts (L929, provided by China Center for Type Culture Collection, Wuhan University, Wuhan, China) was re-suspended in the culture medium and plated (200 μL/well) into 96-well micrometer plates at 1×10^4 cells/well. The plates were incubated at 37 °C in a 5% CO_2 atmosphere for 24 h. The medium was then replaced by 50 μL/well sterilized extract, using the culture medium itself as a control. After incubating for 1, 3, 5 or 7 days, the cells were treated with 20 μL/well of MTT (5 mg/mL in PBS filtered for sterilization), to reach a final concentration of 0.5 mg MTT per mL, and incubated for a further 4 h at 37 °C in a 5% CO_2 atmosphere. At this stage, the MTT was removed and 200 μL/well of dimethyl sulfoxide (DMSO) was added to dissolve the formazan crystals. The plates were placed in an incubator at 37 °C to shake for 10 min. The absorbance values were read in triplicate against a reagent blank at a test wavelength of 570 nm (Tecan GENios, Tecan Austria GmbH, Salzburg, Austria) [33]. Cell viability was calculated using the following equation:

$$\text{Cell viability} = (A_{\text{test}}/A_{\text{control}}) \times 100\% \quad (3)$$

where A_{test} and A_{control} were the absorbance values of the test and control groups, respectively.

Cell culture on films and observation of cell morphology

The ZE/PPI-*n* ($n = 0$, 10, 30, 50, 70, 90 or 100) films were cut into 1.5 cm diameter circles that were sterilized by autoclaving without soaking films in water in order to minimize the dissolution of glycerol and protein components, and then transferred to the bottom of 24-well plastic culture plates. The L929 cell suspension (100 μL) was added to each triplicate sample. After 3–4 h, the samples were supplemented with 1 mL of DMEM containing 10% FBS. The L929 cells, with a cell density of 2×10^5 cells cm^{-3}, were cultured in an incubator for 3 days. The films with cultured L929 cells were then washed with PBS, fixed for 2 h in 2.5 wt% glutaraldehyde, and post-fixed for 2 h in 1 wt% OsO_4. After washing again with PBS, they were progressively dehydrated in ethanol and then dried in super-critical CO_2. These super-critical CO_2 dried films were mounted on stubs and coated with gold for SEM observation at an accelerating voltage of 20 kV. The cell density on the surfaces of the films was determined from the SEM photographs by HLPAS-1000 High Resolution Imaging Treatment System (HLPAS-1000, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China).

Results and discussion

Structure and physical properties

FTIR analysis

The FTIR spectra of the ZE/PPI-*n* films are illustrated in Fig. 1a. The spectra of all the films were very similar due to the chemical similarities between zein and pea protein

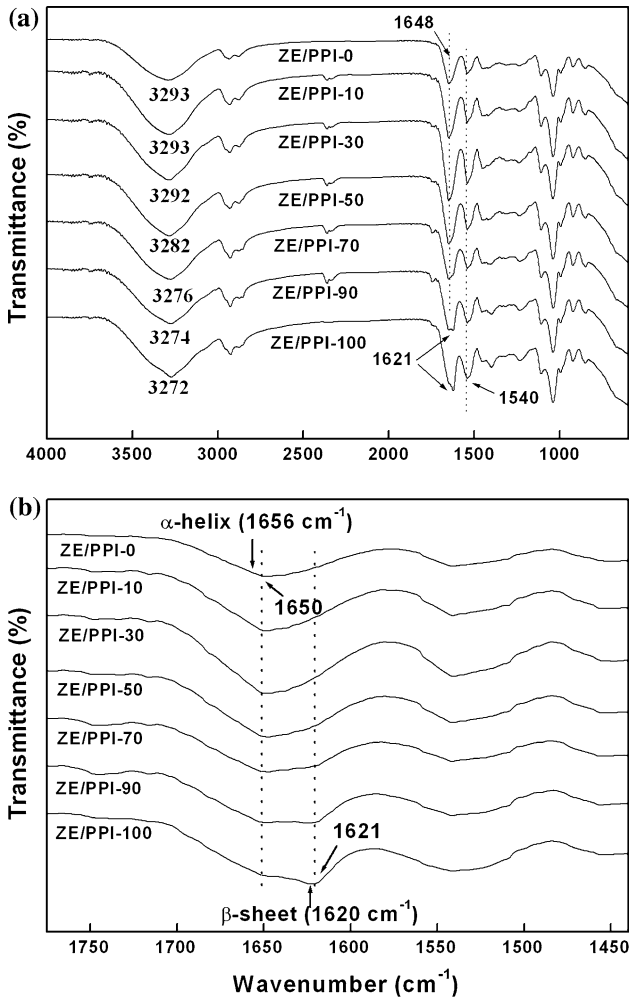

Fig. 1 FT-IR spectra of ZE/PPI-*n* ($n = 0$, 30, 50, 70 and 100) films

isolate, which both consist of peptides. The spectral peaks at 3272–3293 cm^{-1} were attributed to the stretching of $–NH_2$ and –OH groups in protein and glycerol. The peak at 1540 cm^{-1} corresponded to the characteristic absorptions of amide II ($\delta_{N–H} + \nu_{C=O}$) in protein [34]. Peaks at 1621–1650 cm^{-1} were assigned to the characteristic absorptions of amide I ($\nu_{C=O}$) in protein [35].

In spite of the similar spectra presented for all of the samples, close examination of some of the bands, particularly the amide I mode which had the strongest signal, indicates some relevant differences. Figure 1b shows a high magnification of the amide I mode for the samples in Fig. 1a. As reported in previous work [36–38], the α-helix or β-sheet of the amide I mode yielded a rather symmetric peak positioned at 1656 cm^{-1} or a band at 1620 cm^{-1}, respectively. In Fig. 1b, the neat zein film (ZE/PPI-0) had a peak around 1650 cm^{-1}, suggesting that the hot pressed zein film was predominantly of the α-helix secondary structure. The neat pea protein film (ZE/PPI-100) exhibited a band at 1621 cm^{-1}, revealing the existence of a β-sheet secondary structure. The ZE/PPI composites, except for ZE/PPI-90, showed an amide I mode similar to that of the zein film indicating that, in most of the ZE/PPI-*n* films, the secondary structure of the two proteins (zein and pea protein isolate) was mainly α-helix.

Morphology of the original materials and the films

The SEM photographs of the original ZE and PPI particles and the ZE/PPI-*n* films are shown in Fig. 2. The original ZE particles (Fig. 2a) have an irregular polyhedron-like structure and the PPI particles (Fig. 2b) have a ball-like structure. After extrusion and hot press processing, the original polyhedron- or ball-like structures were not observed in the neat ZE (Fig. 2c) or PPI (Fig. 2i) films indicating that, due to their thermoplasticity, both the ZE and PPI particles had been plasticized by glycerol. Cross-sections of the composite films (Fig. 2d–h) exhibited compact structures with small pores and a fractured imprint. The composite films had rougher cross-sections than the neat ZE (Fig. 2c) and PPI (Fig. 2i) films. In general, serious phase separation will inevitably occur between components with obviously different solubilities or hydrophilicities when the casting/evaporation method is used to prepare composite films. No obvious aggregate domains or particle-like structures were observed in the PPI/ZE composites (which consisted of hydrophobic zein and hydrophilic pea protein isolate) indicating that the molecular chains of the two kinds of protein interacted and adhered to each other due to the plasticization of both ZE and PPI by glycerol, as well as the high shear force and high temperature of the extrusion and hot press process.

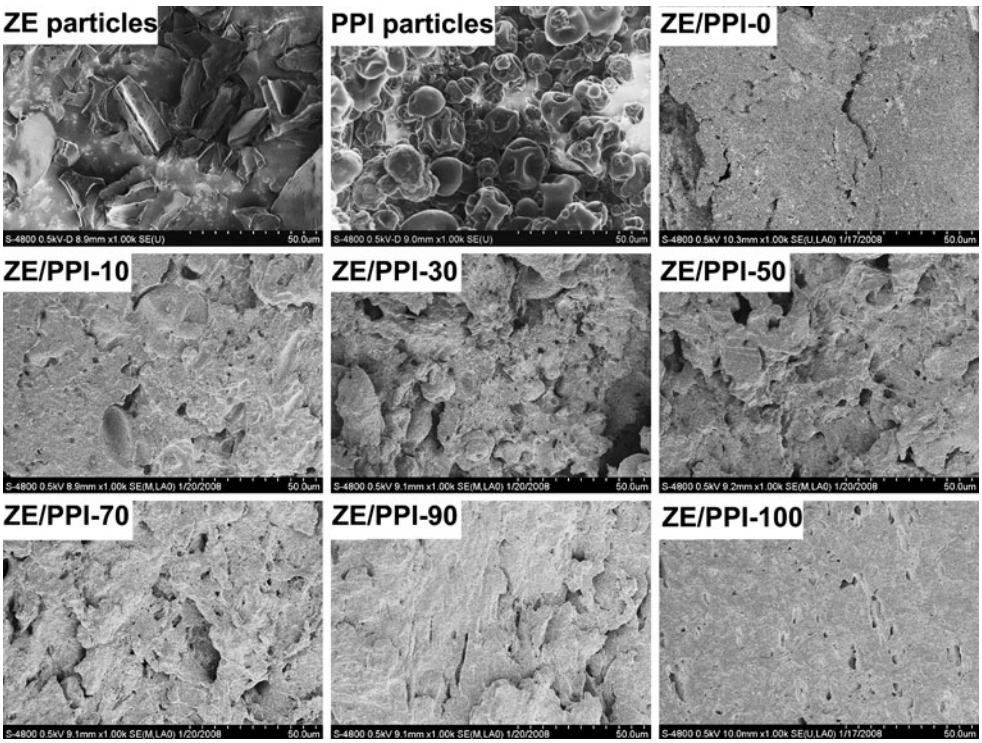

Fig. 2 SEM images of raw materials (ZE particles and PPI particles) and ZE/PPI-*n* (n = 0, 10, 30, 50, 70, 90 and 100) films

This suggests that extrusion and hot pressing is a more suitable method than casting for fabrication of zein-based films or scaffolds for biomedical applications.

Thermogravimetric (TG) and differential thermogravimetric (DTG) analyses

The TG and DTG curves for the films are shown in Fig. 3a, b. The TG and DTG curves show the weight loss of the material as it is heated. The gradual weight loss of the samples was due to the evaporation of water and glycerol, and to the dehydration and decomposition of the polymers [39]. It was reported in a previous work [40] that the decomposition peak of zein was located at 302 °C. In our work, the decomposition peak of zein in ZE/PPI-0 occurred at 317 °C perhaps because of a different source or different molecular weight of the zein. Due to the incorporation of glycerol to zein and the moisture absorption of the film before testing, the DTG curve of ZE/PPI-0 became more complex than that reported by Woods et al. [40]. It can be observed that the DTG curve of ZE/PPI-100 looked very similar to that of ZE/PPI-0 in the temperature ranges from 30 to 145 °C and from 280 to 480 °C. In this case, the peaks in the DTG curves of both ZE/PPI-0 and ZE/PPI-100 have the same positions though they have different peak heights. This means that ZE/PPI-0 and ZE/PPI-100 exhibited almost the same decomposition behaviour due to their chemical similarity, but had a different percent decomposition at the same temperature. In the DTG curves of all samples, a broad peak was observed from 130 to 280 °C. This was mainly due to evaporation of glycerol from the protein composites. All of the composite films showed a T_{max} (corresponding to the temperature at maximum rate of loss in mass) in the range of 280–360 °C. All of these peaks were located at the same position, suggesting that the decomposition peak of zein and pea protein isolate overlapped in the composite films. From the TGA results, it was found that the protein components had a maximum rate of mass loss at 280–360 °C. This suggested that the composites could be sterilized by autoclaving without being soaked into water at a temperature of 121 °C with minimum loss of glycerol.

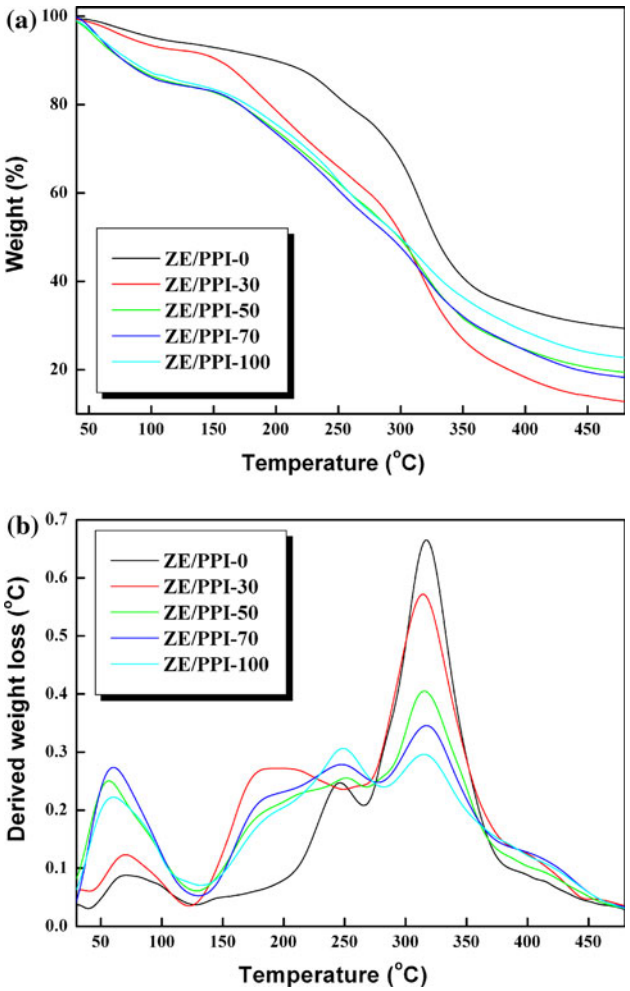

Fig. 3 TGA (**a**) and DTG (**b**) curves of ZE/PPI-*n* ($n = 0$, 30, 50, 70 and 100) films

Mechanical properties of the films

Biodegradability is the main feature of protein-based films [41]; however, the mechanical properties of biopolymers are equally important for maintaining structural integrity, especially when they are used in vitro or in vivo in high moisture or liquid environments [42]. Mechanical properties of the ZE/PPI-*n* films, including tensile strength (σ_b), elongation at break (ε_b) and Young's modulus (E), are shown in Fig. 4. The σ_b, ε_b and E values of the neat zein film (ZE/PPI-0) were 8.2 MPa, 2.4% and 5961 MPa, respectively. The σ_b, ε_b and E values of the neat PPI film

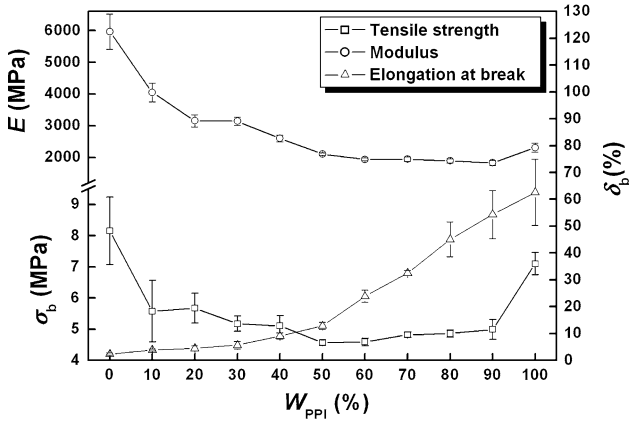

Fig. 4 Tensile strength (σ_b), elongation at break (ε_b) and modulus (E) of ZE/PPI-*n* ($n = 0$, 10, 30, 50, 70, 90 and 100) films at a relative humidity of 46% for 1 week

(ZE/PPI-100) were 7.1 MPa, 62.6% and 2310 MPa, respectively. The ZE film exhibited higher tensile strength and modulus than the PPI film due to the rigidity of zein molecular chains; however, the elongation at break of the PPI film was 25 times higher than that of the ZE film. For composite films containing 10–90 wt% PPI (W_{PPI}), the tensile strength changed slightly from 4.6 to 5.6 MPa, the modulus decreased gradually from 4051 to 1834 MPa and the elongation at break increased quickly from 3.9 to 54.3%. The tensile strength values of the composite films were lower than those of the neat ZE/PPI-0 and ZE/PPI-100 films, an indication of the poor compatibility between the two proteins possibly due to the nature of their different hydrophilicities. The elongation at break of the composite films, however, increased with an increase in PPI content, and modulus increased with an increase in ZE content. This indicated that PPI contributed greatly to the elongation at break and ZE to the modulus of the composites, thus the flexibility of zein can be improved by incorporation of pea protein isolate into zein.

Weight change and weight loss of films soaked in water

The weight change (W_{change}) of films soaked in water for 7 days is shown in Fig. 5. Without a doubt, the net weight change of films depends upon the components' absorption of water and their dissolution to water. In general, water absorption of a hydrophobic material soaked in water should be lower than that of a hydrophilic material. As a result, the weight gain of the hydrophilic material should be higher than that of the hydrophobic material. However, if there are some components in the material which may be dissolved by water, the weight change of the material will be complex. In this work, the change in weight of films soaked in water was a complex process involving water absorption of the films, dissolution of glycerol from the films into the water and loss of the protein components. The change in film weight can be divided into three stages (shown in Fig. 5) including the quick weight gain (less than 2 h soaking) of the first stage; the gradual weight gain (for ZE/PPI-0, ZE/PPI-10 and ZE/PPI-30) or gradual weight loss (for ZE/PPI-50, ZE/PPI-70, ZE/PPI-90 and ZE/PPI-100) in the second stage, requiring 4–96 h of soaking; and finally an equilibrium point in the third stage when the soaking time was longer than 120 h. As shown in the inset of Fig. 5, the weight of the ZE/PPI-0 and ZE/PPI-10 films increased quickly during the first 1 h of soaking due to the strong water-absorption properties of pea protein isolate and the higher PPI content of the films. However, during the first 2 h of soaking, the weight change of the films did not depend absolutely on the PPI content or the hydrophilicity of the films. This suggests that the interaction between the films and water is a complex process related to the hydrophilicity of the protein composition and the different diffusion rates of both glycerol and protein into water from different ZE/PPI films. The neat ZE film (ZE/PPI-100) continued to gain weight during the 96 h soaking, indicating that the zein film had the ability to absorb water in spite of its strong hydrophobicity. Also, the weight continued to increase, indicating that the rate of glycerol dissolution into the water was slower than the rate of water uptake by the zein film. This slow dissolution of glycerol from the ZE film suggested that, in this case, the interactions between zein and glycerol were strong. In the second stage, the films exhibited a different trend in weight change. The weight of films with a PPI content of 50% or more gradually decreased, while that of other films increased. This further indicated that the incorporation of pea protein isolate into zein obviously increased the hydrophilicity of the composite films. In the third stage, the weight of the films did not change. In this case, it is believed that no glycerol remained in the films because of glycerol's high solubility in water and the long soaking time which allowed for diffusion of glycerol from the films into the water. The weight of the films corresponded to both the water absorption and the loss of protein components. After equilibrium, the weight of films with a higher PPI content was lower than that of films with a lower PPI content indicating that the ratio of soluble/dissoluble components and the hydrophilicity/hydrophobicity of the ZE/PPI-*n* films can be adjusted by changing the weight ratio of zein and pea protein isolate.

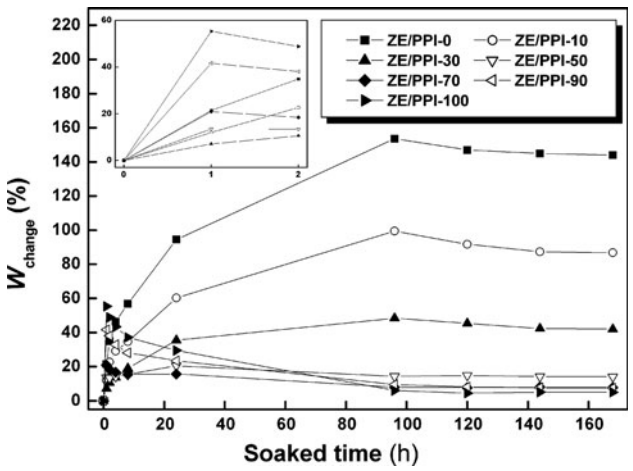

Fig. 5 Dependence of weight change (W_{change}) for ZE/PPI-*n* ($n = 0$, 10, 30, 50, 70, 90 and 100) films on time soaked in water

The film weight loss (W_{loss}), obtained by comparing the dry weights of the films before and after the water-soaking process, is related to the loss of glycerol and protein to the water during the soaking process. The glycerol content is the same in all the films and glycerol will completely dissolve in water after enough time soaking. Therefore, the

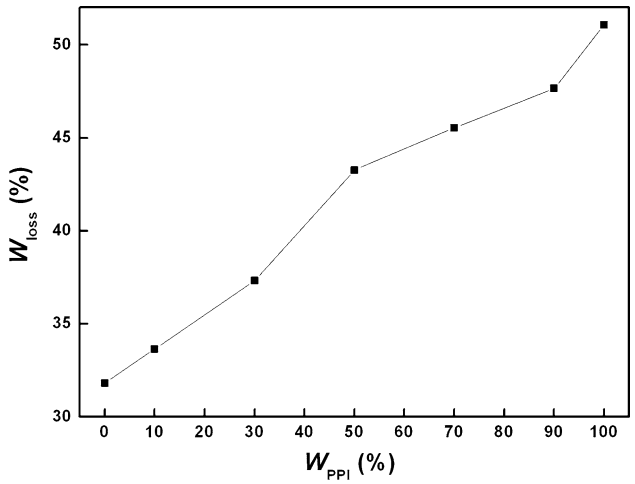

Fig. 6 Weight loss (W_{loss}) of ZE/PPI-*n* ($n = 0$, 10, 30, 50, 70, 90 and 100) films after being soaked in water for 168 h and then dried (*inset* is the weight loss of the films in water from 0 to 2 h)

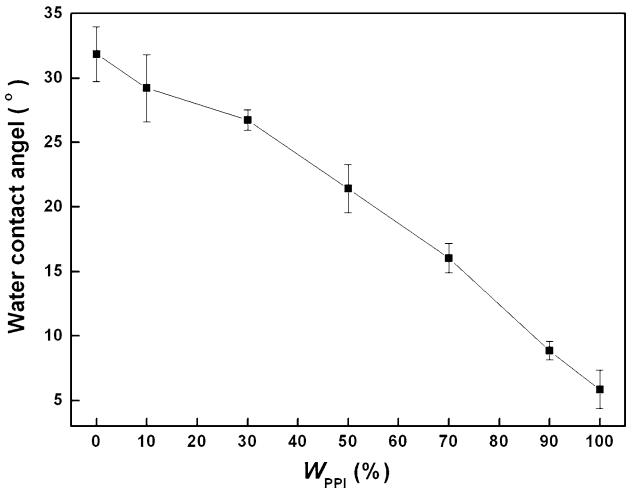

Fig. 7 Water contact angle of ZE/PPI-*n* ($n = 0$, 10, 30, 50, 70, 90 and 100) films

weight loss of the films is directly related to the dissolution of protein in water. In other words, the film weight loss directly reflects the water-resistance of the protein-based films. The percentage W_{loss} for the films soaked in water for 168 h is shown in Fig. 6. The W_{loss} of the neat ZE and PPI films was about 31 and 51%, respectively. This showed that while some zein (1% based on ZE/PPI-0 film, i.e. 1.43% based on the original zein content) in the ZE film dissolved in water, more pea protein isolate (21% based on ZE/PPI-100 film, i.e. 30% based on the original pea protein isolate content) in the PPI film dissolved, assuming all the glycerol (30% based on the whole film, i.e. 100% based on the original glycerol content) dissolved after soaking for a long enough time. Increasing the PPI content from 10 to 90 wt% caused the W_{loss} of the composite films (ZE/PPI-*n*) to gradually increase ($W_{loss} = 33.6$, 37.3 43.2, 45.5 and 47.6%, when $n = 10$, 30, 50, 70 and 90, respectively), due to the higher water solubility of PPI than of ZE.

Contact angle measurement

Contact angle is another parameter related to the surface hydrophilicity of films. Figure 7 shows contact angle values for the ZE/PPI-*n* films at room temperature. All films showed a low contact angle because glycerol was used as the plasticizer. The neat PPI film had a very low initial contact angle of about 5.8°, and the water droplet on the film surface was quickly absorbed due to the strong hydrophilicity and wettability of the PPI film. The neat ZE film exhibited the highest contact angle (31.8°) in the ZE/PPI-*n* film series because it had the highest hydrophobicity. For the composite films, when PPI content increased from 10 to 90%, the contact angle of the composites decreased from 29.2° to 8.9°. This phenomenon corresponds to the high hydrophilic properties of pea protein isolate in comparison with zein's hydrophobic characteristics.

The results of weight change and weight loss of films soaked in water, as well as the contact angles of the films, show that the hydrophobicity of zein in the composite films decreased when pea protein isolate was incorporated and glycerol was used as plasticizer. Tuning the hydrophilicity/hydrophobicity of the film surfaces has important effects on the adhesion and growth of cells on the surfaces, as shown later.

Cytotoxicity and cytocompatibility

MTT assay in extract from ZE/PPI films

The dependence of cell viability of the L929 fibroblasts on cell culture time and on PPI content in the original ZE/PPI-*n* composite films was evaluated by MTT assay and is illustrated in Fig. 8. The viability of cells cultured in the extracts changed with culture time and with PPI content. On the first day, cell viability in the experimental groups (extracts from ZE/PPI-*n* composite films) was very close to that of the control group (culture medium). On the third day, cell viability in all experimental groups was higher than that of the control group; however, on the fifth and seventh days, the ZE/PPI-*n* with a PPI content of 50% or more showed lower cell viability than the control group. It was worth noting that the ZE/PPI-30 film exhibited the highest cell viability during the cell culture process. This indicated that incorporation of 30% PPI into ZE is more beneficial for the proliferation of L929 cells.

Taking the effects of both culture time and PPI content on cell viability into consideration, it was found that pea protein isolate and zein, used separately or in blends, have

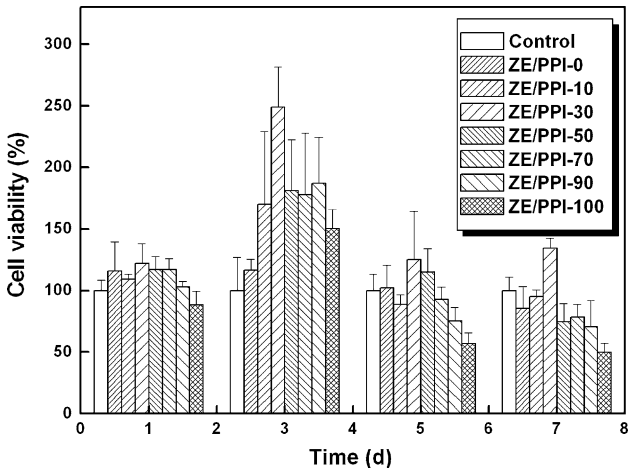

Fig. 8 Cell viability of L929 fibroblasts cultured in extracts from ZE/PPI-*n* ($n = 0$, 10, 30, 50, 70, 90 and 100) films for 1, 3, 5 and 7 days

no side effects on cell proliferation when the cell culture time was not more than 3 days. This indicated that the original zein and pea protein isolates, their blends and small amounts of their hydrolyzates in the culture medium have no obvious cytotoxicity. After a cell culture time of 5 or 7 days, the extracts from films with PPI content of less than 50% were still low- or non-cytotoxic.

Morphology observation of cells cultured on films

SEM photographs of L929 cells cultured for 3 days on the surfaces of ZE/PPI-*n* ($n = 0$, 10, 30, 50, 70, 90 and 100) films are shown in Fig. 9. It was observed that the mainly shuttle or round shaped L929 cells adhered to and grew on the films. The cell density and the percentage of cells that were shuttle or round in shape were calculated from the SEM images and are listed in Table 1. It was observed that

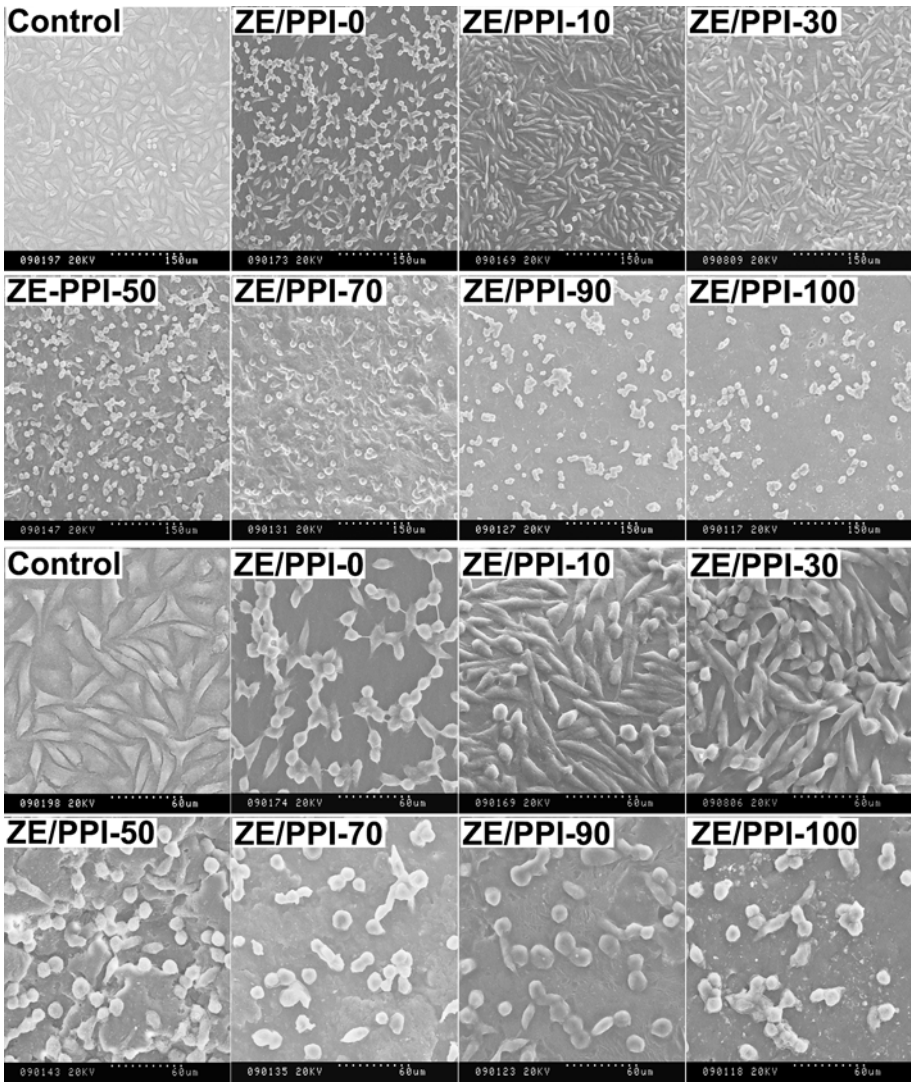

Fig. 9 SEM photographs of L929 cells cultured on the control and on the surfaces of ZE/PPI-*n* ($n = 0$, 10, 30, 50, 70, 90 and 100) films. Scale is labelled on the photographs

cell density decreased from 2307 to 624 mm^{-2} when PPI content increased from 0 to 100% in the original films. The cell density on the control (glass plate) was much lower than that on the ZE/PPI-0 and ZE/PPI-10 films; very close to that on the ZE/PPI-30 and ZE/PPI-50 films; and much higher than that on films with a PPI content greater than 50%. This indicated that a PPI content of 30% or less in the films was beneficial for cell proliferation, while a PPI content of more than 50% prohibited cell proliferation. This is consistent with the results from the MMT assay.

The cell's morphology is another important parameter for evaluating the relationship between cells and materials. As shown in the SEM image of the control, under normal culture conditions most of the L929 cells are shuttle-shaped and expand very well on the surface of the glass plate. If the conditions (including culture medium, temperature and matrix materials) were not suitable, the cells responded by becoming round in shape, shrinking or dying. It is surprising that most of the cells (97.5%) on the surface of the neat zein film (ZE/PPI-0) were round. This may be due to the relatively high hydrophobicity and the compact surface of the zein film, which lead to great difficulties for cell expansion. The morphologies of the cells on the surfaces of the ZE/PPI-10 and ZE/PPI-30 films were very similar to that of the control with 90.0 and 88.0% shuttle shaped on ZE/PPI-90 and ZE/PPI-70 films, respectively, and 95.3% on the control. This indicated that the ZE/PPI-90 and ZE/PPI-70 films were suitable materials for culturing L929 cells due to the appropriate surface hydrophilicity resulting from the addition of PPI. The surface hydrophilicity of the composite films increased with an increase in PPI content in the films; however, the percentage of shuttle shaped cells quickly decreased for the ZE/PPI/50, ZE/PPI-70, ZE/PPI-9- and ZE/PPI-100 films containing more than 30% PPI. For example, the percentage of shuttle shaped cells was only 2.5% on the neat PPI film (ZE/PPI-100), which although had the highest hydrophilicity, suggesting that surface hydrophilicity is not the only factor affecting cell expansion. The cell's morphology depended on both components and on the surface properties of the materials, which were directly related to the preparation and pretreatment process of the films before cell culture. In this work, the ZE/PPI-*n* films used for cell culture were sterilized by autoclaving. The final contents of ZE and PPI, the surface morphology and the surface hydrophilicity/hydrophobicity properties of films might be changed in certain degree by the autoclaving process, which may have effects on the cell's density and morphology on the films. Therefore, the cell behaviours on the surfaces of different films may be due to different chemical compositions in the films (more/less PPI depended on the original PPI content) and the different surface properties of the films (resulted from both the original PPI content and the morphology changes from sterilization process).

As shown in Table 1, the cell density increased with an increase in ZE content and a decrease in PPI content, indicating that zein was more beneficial for cell proliferation than pea protein isolate. However, when ZE content was 100% (ZE/PPI-0), more than 70% of the cells changed their shapes from regular shuttle to round. This suggested that neat zein was not suitable for cell expansion. Most of the cells kept their normal shuttle shape on the films containing 10% and 30% PPI; while most of the cells changed into round shape on the films with 50% or more PPI. Therefore, a certain amount of pea protein isolate played a positive role in cell expansion and a negative role in cell proliferation.

From the point of view of mechanical properties, cytotoxicity, cell density and cell morphology, the ZE/PPI-90 and ZE/PPI-70 films are the most suitable materials for cell or tissue scaffolds among the ZE/PPI-*n* series films. In this case, the incorporation of 10–30% pea protein isolate into zein, not only increased the flexibility of the zein-based films but also modified the surface micro-morphology and enhanced the surface hydrophilicity of the films, resulting in improved cytocompatibility.

Conclusions

The series of ZE/PPI-*n* films with 0–100% PPI was successfully produced by extrusion and hot press processing. The results of tensile testing, weight change in water and water contact angle measurement of the composite films showed that the incorporation of PPI into ZE enhanced the flexibility and surface hydrophilicity of the films. In the cell culture experiments, MTT assay and morphology analysis showed that the L929 cells adhered and grew on the surface of the ZE/PPI-*n* films. The proliferation and expansion of the cells greatly depended on the composition, microstructure and surface hydrophilicity/hydrophobicity of the films, which was tuned by adjusting the weight ratio of ZE and PPI. This work showed that 10–30% PPI in the ZE/PPI-*n* films was beneficial for enhancement in physical properties, modification of surface microstructure and improvement in cytocompatibility of the films resulting in enhanced cell proliferation, cell adherence and cell expansion on the films.

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