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Physical, mechanical and biological properties of poly(ε-caprolactone)–poly(ethylene glycol)–poly(ε-caprolactone) (CEC)/chitosan composite film

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ABSTRACT

In this paper, a series of CEC/chitosan composite films with various CEC contents (0%, 10%, 30%, 50% and 70%) were successfully prepared by casting/solvent evaporation technology in 80% acetic acid solution. The obtained composite films were characterized by Fourier transform infrared spectroscopy (FTIR), X-ray diffractometer (XRD), differential scanning calorimeter (DSC) and scanning electron microscopy (SEM), respectively. Additionally, mechanical properties, *in vitro* water absorption as well as *in vitro* degradation behavior of CEC/chitosan composite films were also investigated. The intrinsic cytotoxicity of extraction fluids of composite films was investigated by MTT assay using Rat osteoblastic cells (ROS cells). Results revealed that the extract fluids of composite films except that of 10% CEC composite film were non-cytotoxic after 24 h incubation. Cell adhesion as well as morphology was analyzed by SEM, which indicated that the ROS cells appeared to adhere well and exhibited a normal morphology on the surface of composite films after 24 h incubation. The obtained composite films might have great potential application in the field of tissue engineering.

1. Introduction

In recent years, there has been increasing interest in optimizing engineered tissue regeneration using biocompatible and biodegradable materials. A series of naturally derived polymers such as gelatin, collagen, chitosan, albumin and starch and chemo-synthetic polymers such as polyethylene glycol, polycaprolactone (PCL), poly(lactic-co-glycolic acid), polyvinyl alcohol and polyurethane have been extensively used in the pharmaceutical and biomedical field (Hutmacher, 2000; Sarasam & Madihally, 2005). Among these polymers, chitosan has gained considerable interest due to its excellent physico-chemical properties such as low-toxicity, good biocompatibility, biodegradability, mucoadhesive, etc. (Jayakumar, Prabaharan, Reis, & Mano, 2005; Kim et al., 2008; Oliveira & Barros-Timmons, 2010; Ravi Kumar, 2000; Shirosaki, Botelho, Lopes, & Santos, 2009; Veleirinho & Delgadillo, 2009). Chitosan, the only cationic polysaccharide in nature, is composed of *N*-acetylglucosamine (GlcNAc) and glucosamine (GlcN) residues. The content of glucoamine in the skeleton is called as the degree of deacetylation (DD). Depending on the source and preparation procedure, the various molecular weight (Mw) from 300 to over 1000 kDa and a DD from 30% to 95% of chitosan could be obtained (Hirano, Tsuchida, & Nagao, 1989; Ilium, 1998). As the product of deacetylation of chitin, chitosan could only be dissolved in some acidic solution when pH value was below 6. A number of studies have been performed on the various chitosan-based implants for tissue engineering (Ravi Kumar, 2000). Okamoto et al. (1995) have reported that the chitosan could influence all stages of wound repair in experimental animal models. Nowadays, clinical trials are being performed on the application of chitosan film/membrane in the wound healing and hemostasis (Azad, Sermsintham, Chandrkrachang, & Stevens, 2004; Bello, Falabella, & Eaglstein, 2001). Additionally, chitosan bears three types of reactive functional groups, an amino group as well as both primary and secondary hydroxyl groups at the C(2), C(3) and C(6), respectively. A number of attempts are being made to modify chitosan, realizing its full potential tissue engineering applications (Azad et al., 2004; Kim et al., 2008). However, less flexibility in regulating the mechanical properties and biodegradation has greatly limited its applications.

On the other hand, many synthetic polymers exhibit well physico-chemical and mechanical properties comparable to chitosan. Poly(ε-caprolactone) (PCL) and poly(ethylene glycol) (PEG) are materials that are biocompatible and have been used in

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several FDA-approved products. PCL is nontoxic and has great permeability. Since Perret and Skoulios firstly prepared a series of copolymers containing PCL and PEG, these copolymers consisted of PCL blocks and PEG blocks have been widely studied (Huang et al., 2008; Peng et al., 2008; Perret & Skoulios, 1972; Zhou, Deng, & Yang, 2003). Our previous studies revealed that the CEC membranes with nanoporous structure and CEC nanofiber made from dichloromethane (DCM) solution had well mechanical strength. However, the obtained CEC membrane has (1) limited bio-regulatory activity, (2) hydrophobicity, (3) neutral charge contribution, and (4) susceptibility to bacteria-mediated degradation. Polymer blending technology is an effective way to obtain new polymeric materials with optimized properties without the complicated process of synthesizing the totally new ones (Li, Chen, Yin, Yao, & Yao, 2007; Rodrigues et al., 2008; Thanpitcha, Sirivat, Jamieson, & Rujiravanit, 2006; Zhou, Song, & Li, 2007). Other advantages of this technology include versatility, simplicity, and inexpensiveness. Blending method has been successfully used to blend some hydrophobic biodegradable polyesters with hydrophilic biodegradable polysaccharides such as poly(ε-caprolactone) (PCL), and polylactide (PLA) with chitosan film to improve its water-resistant properties and alter its mechanical properties (Jiao, Liu, & Zhou, 2007; Wu, 2005). Recently, Sarasam and Madihally (2005) reported that the homogeneous blends of 25%, 50% and 75% PCL compositions could be obtained by dissolving chitosan and 80 kDa PCL in a common solvent of ~77% aqueous acetic acid. Here, we also adopted 80% aqueous acetic acid as the solvent to prepare CEC/chitosan composite films. Films made from various conditions were prepared and characterized.

2. Materials and method

2.1. Materials

Chitosan (with 86% degree of deacetylation (DD)) with ~200 kDa was supplied by Sigma–Aldrich (USA). CEC was successfully synthesized by our previous report and its molecular weight (Mw) of ~40 kDa (the Mw of PEG segment was 4 kDa) was determined by a GPC (Agilent 110 HPLC, USA) (Gou, Dai, et al., 2008; Gou, Qian, et al., 2008). Lysozyme was bought from the Amresco (USA). All other chemicals used in this paper were analytic grade. Distilled water from Milli-Q water system was used to prepare the aqueous solutions.

2.2. Formation of solutions and preparation of CEC/chitosan composite films

The casting/solvent evaporation technology was employed to prepare the composite films of chitosan and CEC. Initially, chitosan (1 g) was dissolved in 80% acetic acid solution (99 ml) under magnetic stirring for 48 h at room temperature. The resulting chitosan solution was filtered and stored at 4 °C for further application. CEC solution was obtained by dissolving calculated weight of CEC into a certain glacial acetic acid. Subsequently, 20 ml of film-formation solutions, regardless of weight ratio of CEC/chitosan (0%, 10%, 30%, 50% and 70%), were poured into a glass Petri dish and dried at 60 °C for 1 day. The obtained series of films were neutralized by 1 M NaOH solution for 2 h and washed with distilled water to neutral (pH = 7). Finally, the wet films was wiped with a filter paper to remove the excess water present on the surface of the membrane and allowed to dry at room temperature for 24 h. The obtained composite films (CEC%, w/w) named 0% CEC composite film, 10% CEC composite film, 30% CEC composite film, 50% CEC composite film and 70% CEC composite film, respectively.

2.3. Characterization of blend membrane

2.3.1. Fourier transform infrared spectroscopy (FTIR)

FTIR (KBr) spectra were performed at room temperature using NICOLET 200SXV Infrared Spectrophotometer (USA). The characteristic absorption bands of the composite films and polymers were detected at wavenumbers ranging from 500 to 4500 cm^{-1}.

2.3.2. Crystallographic assay

X-ray diffraction spectra of various composite films were obtained on a X-ray diffractometer (DX-2000, DanDong Fangyuan Instrument Company, China) using CuKα radiation.

2.3.3. Thermal properties

The thermal properties of various composite films were characterized by a differential scanning calorimeter (DSC, NETSCZ 200, Germany). The purified and dried samples were used for DSC test. Samples were first heated from 20 °C to 100 °C under nitrogen atmosphere at a heating rate of 10 °C/min, and reheated to 100 °C at the same rate after quenched to 20 °C, at last sample was cooled to 20 °C again at the cooling rate of 10 °C/min.

2.3.4. Tensile properties

Rectangular specimens of the CEC/chitosan composite films with dimensions of 40 mm × 5 mm × 0.06–0.10 mm were tested by a universal mechanical testing instrument (Instron-5567, Instron Corp., USA) at room temperature and relative humidity of 50%. The tensile strength and the elongation were evaluated at a displacement rate of 20 mm/min with 20-mm gauge length. All results were the mean values of five specimens.

2.4. Water absorption study

Water absorption of composite films was detected by weighing the film pieces after placing in pH 7.4 phosphate buffer solution. Each film was divided into portions of 1 cm^2 (1 cm × 1 cm) and cut, weighed and placed in buffer solution for 24 h. Finally, the films were taken from the medium and weighed after removal of the surplus surface water using filter paper. The percentages of water absorption were calculated by the following Eq. (1):

$$\text{water absorption}\,(\%) = \frac{W_{24} - W_0}{W_0} \times 100 \quad (1)$$

where W_{24} is the weight of wet film at time 24 h and W_0 is the original film weight at zero time, respectively.

2.5. In vitro degradation test

According to our pervious description (Li et al., 2010), *in vitro* degradation behavior of composite films (1 cm × 1 cm) was determined in 5 ml phosphate-buffered solution (PBS, pH = 7.4) at 37 °C containing 1.5 μg/ml of lysozyme. The concentration of lysozyme was chosen to correspond to the concentration in human serum. Briefly, the calculated weight films were incubated in the lysozyme solution with gentle agitation for periodical study. Lysozyme solution was refreshed daily to ensure continuous enzyme activity. Samples were removed from the medium at predetermined time (7, 14, 21 and 28 days), and rinsed with distilled water, finally dried under vacuum and weighed. The degree of *in vitro* degradation was calculated by the weight loss:

$$\text{weight loss}\,(\%) = \frac{W_0 - W_t}{W_0} \times 100$$

where W_0 is the dry weight before degradation test and W_t is the dry weight at predetermined time t.

2.6. In vitro cell culture studies

2.6.1. Cytotoxicity screening

According to the previous report of Oliveira et al. (2006), MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) test was used to determine the cytotoxicity of CEC/chitosan composite films leachables that might result from the processing methodology used to obtain the chitosan films and/or leachables of the polymeric component of the composite films. Rat osteoblastoid cells (ROS cells, ATCC, USA), were cultured in basic medium at 37 °C and 5% CO_2: Dulbecco's Modified Eagle's Medium (DMEM, Sigma–Aldrich, USA) without phenol red supplemented with 10% foetal bovine serum (FBS, Sigma–Aldrich, USA), and antibiotic–antimycotic (1% A/B, Sigma–Aldrich, USA) solution containing 10,000 units ml^{-1} penicillin G sodium, 10,000 mg ml^{-1} streptomycin sulphate and 25 mg ml^{-1} amphotericin B as Fungizones in 0.85% saline. First, ROS cells were cultured in DMEM to achieve with the cell concentration at 1×10^5 cells ml^{-1}, and then seeded onto 96-well plates. ROS cells were incubated for 24 h with a series concentrations of CEC/chitosan composite films extracts (25%, 50%, 75% and 100%, respectively). All results were the mean values of three specimens.

2.6.2. Evaluation of ROS cell adhesion and morphology

Cell adhesion, morphology, and spatial distribution were observed by a scanning electron microscopy (SEM). Cell-constructs after 24 h incubation first were washed with a phosphate-buffered saline (PBS, pH = 7.4) solution and fixed in 2.5% glutaraldehyde at 4 °C overnight. Subsequently, the cell-constructs were dehydrated using a graded series of ethanol (30%, 50%, 70%, 80%, 90%, 95%, 100%) for 10 min. Finally, the cell-construct was dried in a vacuum oven for 4 h. Afterward, the cell-construct was sputter coated with gold

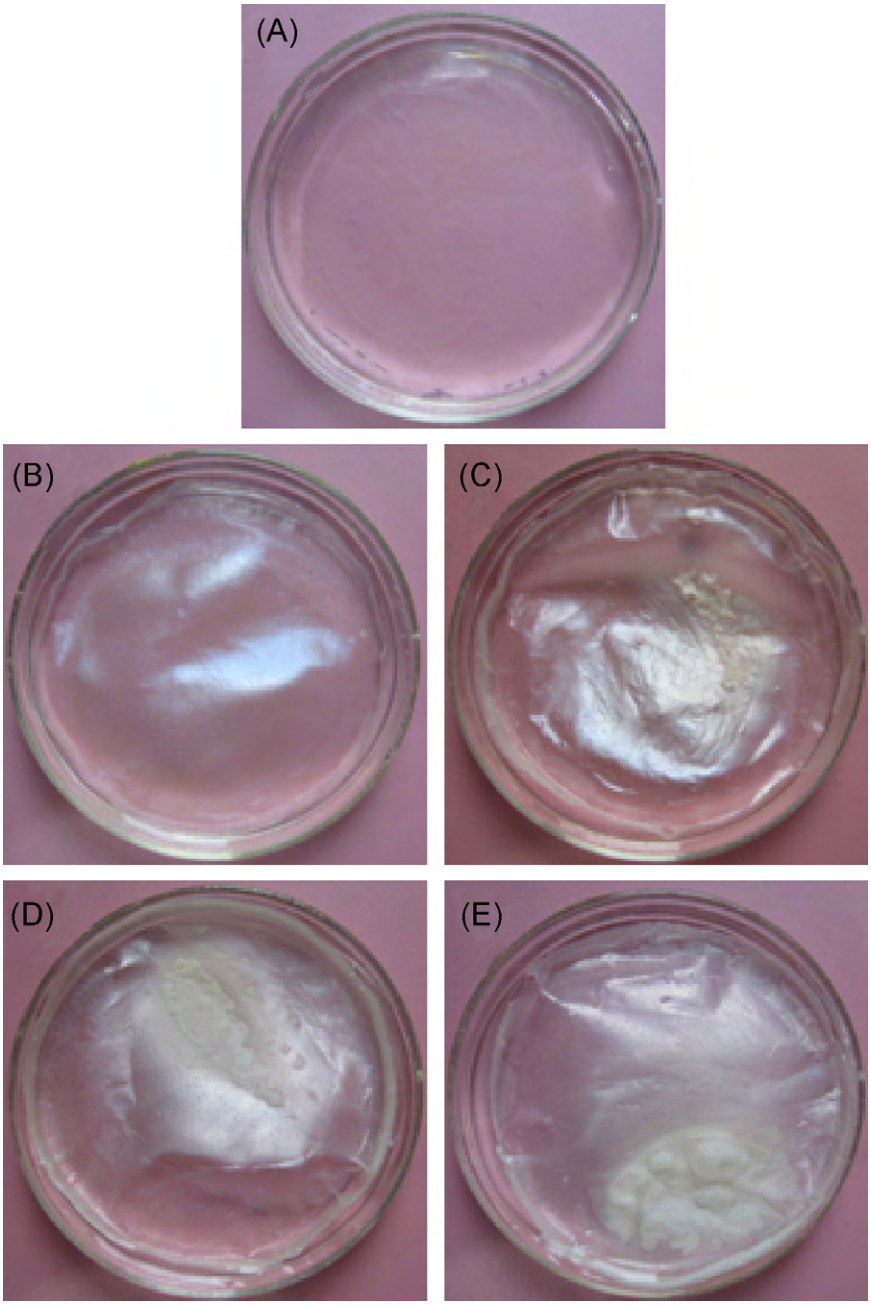


Fig. 1. Appearance of CEC/chitosan composite films made from various CEC concentrations: 0% (A), 10% (B), 30% (C), 50% (D) and 70% (E).

and observed by scanning electron microscopy (JSM-5900LV, JEOL, Japan).

3. Results and discussion

3.1. Appearance of blend membrane

The data has been available in the literature for the physical, mechanical and biological properties of chitosan films and chitosan composite films. However, blending CEC with chitosan in single phase of 80% aqueous acetic acid solution was first reported and characterized. All the prepared films had a uniform thickness of 20–60 μm. It is well know to us that the chitosan powder is white or white-like. However, the prepared blank chitosan film is transparent in the water solution as shown in Fig. 1A. CEC membrane made from 80% acetic acid solution did not have structural integrity, unlike the film made from only chitosan (data not shown). This might be attributed to the phase behavior of CEC in 80% acetic acid solution which is in accordance with the report of Sarasam and Madihally (2005). As depicted in Fig. 1, with the increase of CEC content in composite film, the transparence decreased accordingly.

3.2. FTIR analysis

FTIR spectra of CEC/chitosan composite films are shown in Fig. 2A. The characteristic absorption bands of chitosan has been described previously (Jayakumar et al., 2005; Li et al., 2010) and they corresponded to: $-NH_2$ (1650 and 1560 cm^{-1}), C–O–C (1114 cm^{-1}) and the band at 3480 cm^{-1} belonged to the stretching vibrations of hydroxyl groups. According to our previous studies (Liu et al., 2008), the characteristic absorption bands of CEC corresponded to: –COO (1723 cm^{-1}), C–O–C (1142 cm^{-1}) and the band at 3508 cm^{-1} belonged to the stretching vibrations of hydroxyl groups. These characteristic absorption bands of chitosan and CEC were also observed in all of the composite films although with varying intensities based on their composition. Characteristic bands of chitosan were more distinguishable in 10% and 30% CEC composite films, yet those of CEC were better expressed in 50% and 70% CEC composite films. Sarasam and Madihally (2005) have reported that the presence of chitosan in PCL composite film could interfere the crystallization of PCL film resulting in the alteration of properties of composite film. The amine group and hydroxyl group of chitosan could potentially form a hydrogen bond with the carbonyl group of ester groups in the chain of PCL (Fig. 2B). In such a case, the two characteristic bands to amine group of chitosan with weaken intensity would shift the lower wavenumbers (from 1650 to 1648 cm^{-1}) as shown in Fig. 2A. These changes of FTIR spectra of composite films indicated that there is no detectable chemical bonding between chitosan and CEC but presence of possible molecular interaction between chitosan and CEC.

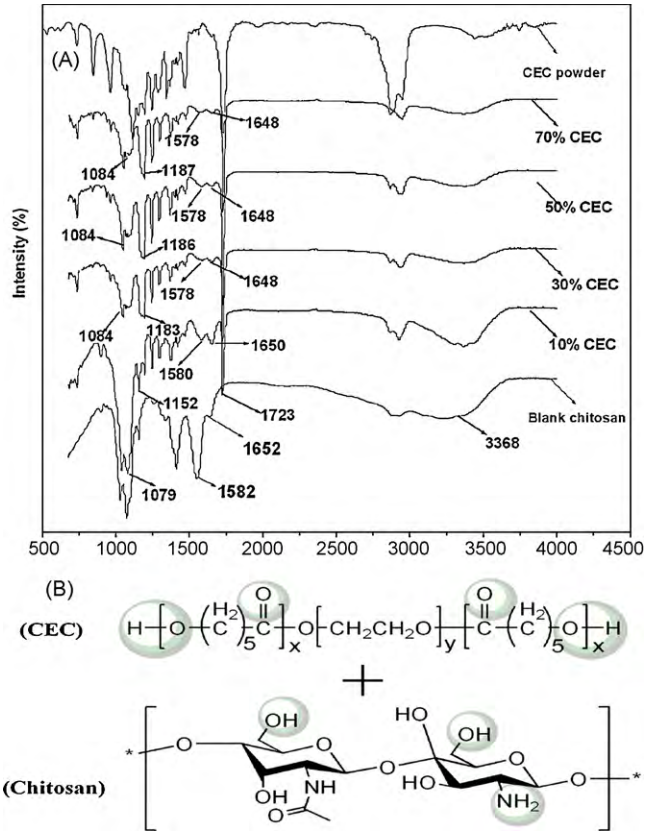


Fig. 2. Molecular interaction between chitosan and CEC. (A) FTIR spectra of chitosan/CEC blends and (B) possible bond interaction between chitosan and CEC.

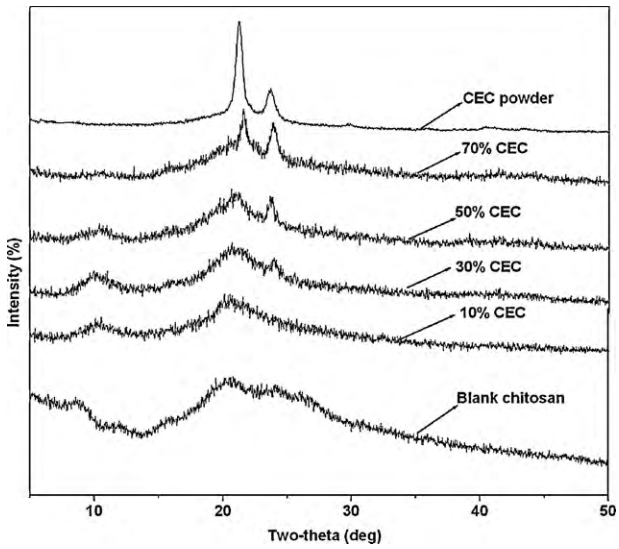


Fig. 3. XRD patterns of chitosan, CEC and composite films with various CEC contents (10%, 30%, 50% and 70%).

3.3. Changes of crystal structure

The crystallographic structures of chitosan and CEC/chitosan composite films were determined by XRD. As presented in Fig. 3, chitosan, which is a semi-crystalline polymer, exhibits a reflection peak at about 20° and a relatively weak reflection at 10°, which are assigned to two different crystal forms (Jayakumar et al., 2005; Kong et al., 2010). Both the characteristic peaks of chitosan appeared in 10% CEC, 30% CEC and 50% CEC composite films except 70% CEC composite film. The diffraction pattern of CEC showed sharp and well-defined characteristic peaks at 21.5° and 23.5° (Gou, Dai, et al., 2008; Gou, Qian, et al., 2008). These peaks were more distinguishable in 50% and 70% CEC composite at the same angles with slightly reduced intensity. 50% CEC blending showed all peaks of CEC as well as the chitosan peak at 10°, although with much reduced intensity. The absence of any additional peaks or shift in the diffraction angles indicated that the crystal structure of chitosan and CEC are not changed in the composite films. The crystal structure of composite films was influenced by the dominant component; that is, the spectrum of 10% and 30% CEC composite films was similar to that of chitosan, whereas the spectrum of 50% and 75% CEC composite films was similar to that of CEC, suggesting that chitosan and CEC coexist as the separate phases in the composite films.

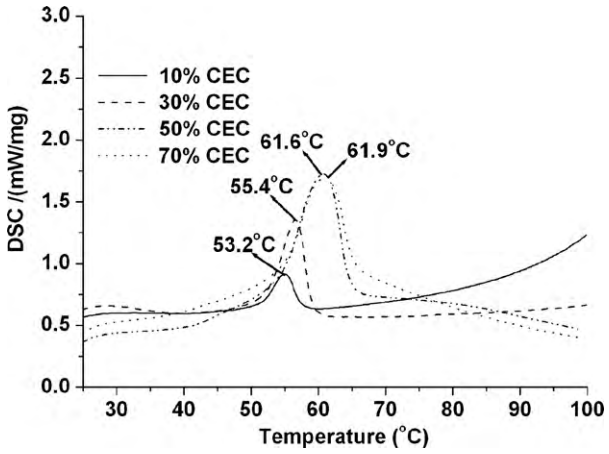
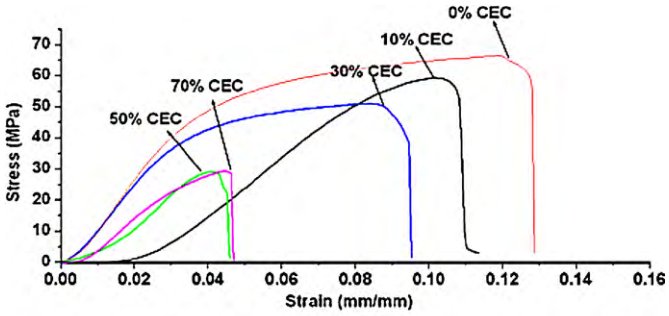


Fig. 4. Differential scanning calorimeter (DSC) spectra of CEC/chitosan composite films with various CEC contents (10%, 30%, 50% and 70%).

3.4. Thermal properties

The depression of melting point of a crystalline polymer blended with other polymers provides information about their miscibility (Sarasam & Madihally, 2005). Both chitosan and CEC are crystalline polymers; pure CEC melts at about 62 °C and its glass transition temperature is around −60 °C (Gou, Dai, et al., 2008; Gou, Qian, et al., 2008). On the other hand, chitosan undergoes thermal degradation at 270 °C prior to melting. Thus, the melting point of CEC was monitored to understand the miscibility of the two polymers. As depicted in Fig. 4, the melting temperature (T_m) of composite films increased from 53.2 °C to 61.9 °C as the CEC content increasing from 10% to 70%. The significant shift of T_m in various blending films indicated that the crystal structure of CEC was greatly affected by the current method of blending. Meanwhile, the changes of T_m with respect to that of pure CEC also suggested the miscibility and interphase interaction between the components of a polymer blend or composite (García Cruz, Coutinho, Mano, Gómez Ribelles, & Salmerón Sánchez, 2009). This result was in accordance with the conclusion obtained by FTIR and XRD analysis.



Samples	Break strength (MPa)	Break strain (%)	Elastic modules (MPa)
0%CEC	71.63±3.15	14.32±2.96	2452.35±178.24
10%CEC	61.41±2.12	12.14±1.67	1625.34±102.34
30%CEC	50.35±3.12	9.35±1.34	1139.89±98.75
50%CEC	25.45±2.23	5.02±1.29	1150.34±143.45
70%CEC	26.56±3.35	5.10±1.48	1022.23±102.61

Fig. 5. Tensile properties and stress–strain curves CEC/chitosan composite films with various CEC contents (0%, 10%, 30%, 50% and 70%).

3.5. Film mechanical properties

Mechanical property is an important property for polymer hybrid membranes/films which is potentially used in tissue engineering. In order to investigate the effect of CEC concentrations on the mechanical property of the CEC/chitosan composite films, tensile experiments were performed. The tensile strength, elongation rate and Young's modulus of the composite films were obtained and listed in Fig. 5. CEC dissolved in glacial or dilute acetic acid did not form structural integrity film at 60 °C. This could be explained by the highly crystalline nature of CEC dissolved in acetic acid, which is similar to the report of Sarasam and Madihally (2005). As presented in Fig. 5, the tensile strength of the film decreased from 71.63 ± 3.15 MPa for blank chitosan film to 26.56 ± 3.35 MPa for 70% CEC composite film. Interestingly, the tensile strength

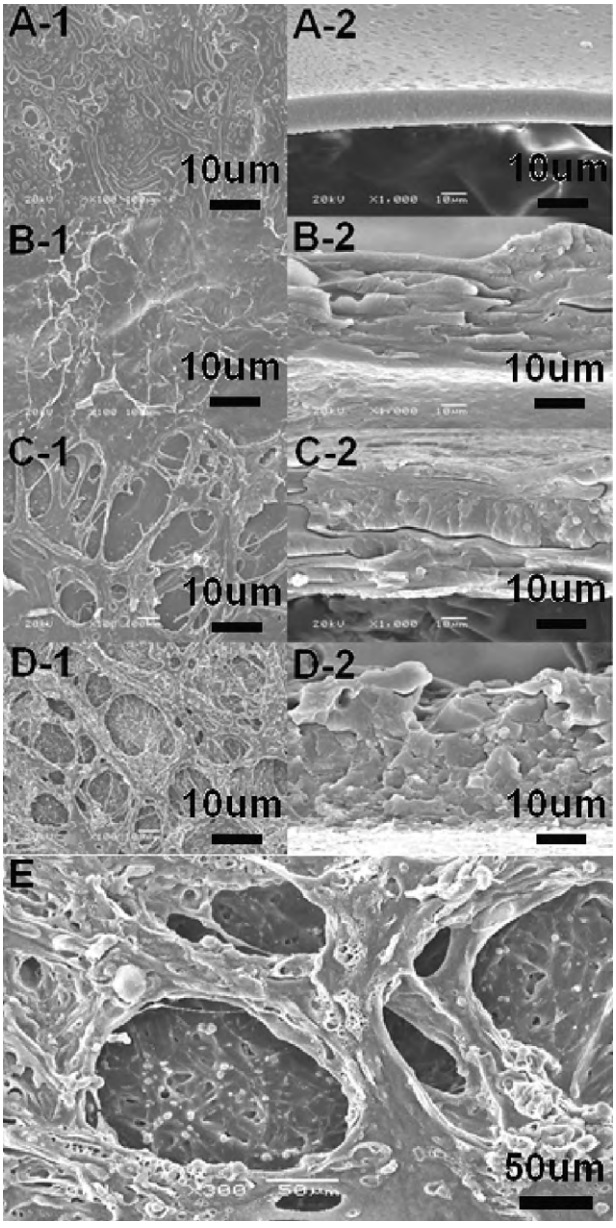


Fig. 6. SEM micrographs of the CEC/chitosan composite films with various CEC contents: (A-1) surface of 10% CEC composite films; (A-2) cross section of 10% CEC composite films; (B-1) surface of 30% CEC composite films, (B-2) cross section of 30% CEC composite films; (C-1) surface of 50% CEC composite films, (C-2) cross section of 50% CEC composite films; (D-1, E) surface of 70% CEC composite films, (D-2) cross section of 70% CEC composite films.

decreased greatly as the CEC fraction increased from 10% to 50%, but there was no significant difference in tensile strength between 50% CEC composite film and 70% CEC composite film. This might be explained by that the serious phase separation of chitosan and CEC in the composite films was generated at a high CEC content (>50% CEC) (Sarasam, Krishnaswamy, & Madihally, 2006; Sarasam, Samli, Hess, Ihnat, & Madihally, 2007). Meanwhile, Sarasam et al. (2006) also suggested that drying temperature as well as chloroform annealing could possibly alter the mechanical properties of PCL/chitosan blend film. Therefore, the mechanical properties of CEC/chitosan composite film could be well tailored by well adjusting these parameters. Based on these results, it showed that the tensile properties of composite films were greatly depended on the composition and processing conditions such as drying temperature, humidity, etc. Therefore, there is need for a better processing technology that could minimize separation of these polymers.

3.6. Morphological analysis

Recently, Sarasam and Madihally (2005) reported the possibility of blending chitosan and PCL in a single phase using a unique acetic acid solution without complex chemical modifications intending for the tissue engineering. However, the surface of prepared blend PCL/chitosan blend film was smooth which is not conducive to cell adhesion. Herein, we developed a series of composite films with honeycomb structure based on chitosan and CEC intending for the tissue engineering. Surface and cross section of composite films were studied by SEM. According to Fig. 6, we could find that the roughness of composite film increased no matter at surface or cross section of composite films with the increase of CEC content from 10% to 70% in composite films. Specifically, the roughness increased significantly as the CEC content was above 50%. This might be attributed to a serious phase separation of these two polymers resulting in the discontinuous structure of composite film, which has been demonstrated by mechanical test, FTIR analysis, DSC test and XRD test. As the CEC content in the composite film was above 50%, the obvious pore structure as well as the spherical particles on the film surface was observed at 100× and 300× magnification (Fig. 6D and E), which might be more conducive to cell adhesion suitable for tissue engineering. These particles might be composed of the CEC, which is the similar to the report of Sarasam et al. (2007). And these particles were not hollow and the arrangement seemed random, which could be clearly visible in the 70% CEC composite film (Fig. 6E). However, although the obvious pore structure and spherical particles were observed in the surface of 50% and 70% CEC composite films, the cross section of these composite films did not show any pore structure, unlike the interconnected open pore surface structure.

3.7. Water absorption study

As a film/membrane for tissue engineering, besides the pore structure, the water absorption of film also played an important role in the tissue engineering which not only affects its morphology and structure but also affects the ingrowing cells (Ma, Wang, He, & Chen, 2001). According to the previous report (Li et al., 2010), water absorption was measured by weighting the composite films before and after placing in the water solution. As presented in Fig. 7A, water absorption varying from 220% to 70% showed the great dependence on the content of CEC in the composite films. Water absorption decreased significantly with the CEC content increasing from 0% to 70%. Although absence of the pore structure to 0%

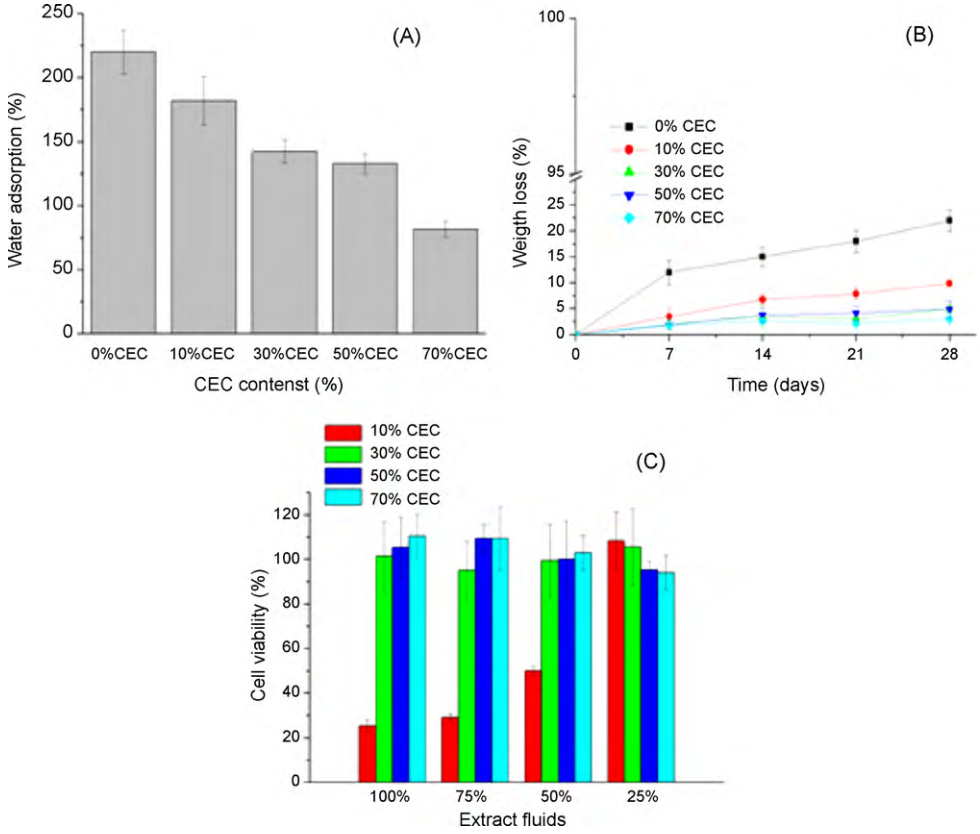


Fig. 7. Water absorption (A) and *in vitro* degradation profiles (B) of composite films with various CEC contents (0% CEC, 10% CEC, 30% CEC, 50% CEC and 70% CEC); cytotoxicity screening for CEC/chitosan composite films (C): ROS cells were incubated with different concentrations of leachables obtained from composite films with various CEC contents (10% CEC, 30% CEC, 50% CEC and 70% CEC).

CEC blend film, the highest water absorption of about 220% could be gained among these composite films. As we all known that chitosan is hydrophilic polymer bearing primary amine ($-NH_2$) and hydroxyl group (–OH), which can not only increase its affinity to water but also form hydrogen bonds with water (Li et al., 2010; Nettles, Elder, & Gilbert, 2002). As the blending with hydrophobic polymers (CEC), due to the possible interaction (hydrogen bond) between these polymers (identifying by FTIR analysis), the hydrophilicity of chitosan decreased accordingly, thus the water absorption decreased accordingly. All these results suggested that water absorption could be well modulated by changing the composition of composite films.

3.8. In vitro degradation behavior

Although a number of *in vitro* degradation test of chitosan and its derivatives have been performed in past decade, most of the studies were carried out with accelerated conditions using low pH for optimum lysozyme activity as well as high enzyme concentrations (Freier, Koh, Kazazian, & Shoichet, 2005; Lee, Ha, & Park, 1995). To our knowledge, long-term *in vitro* degradation studies of CEC/chitosan blend film at physiological pH with enzyme concentration correspond to the concentration in human serum (1.5 μg/ml) have not yet been published. It is well known that, in

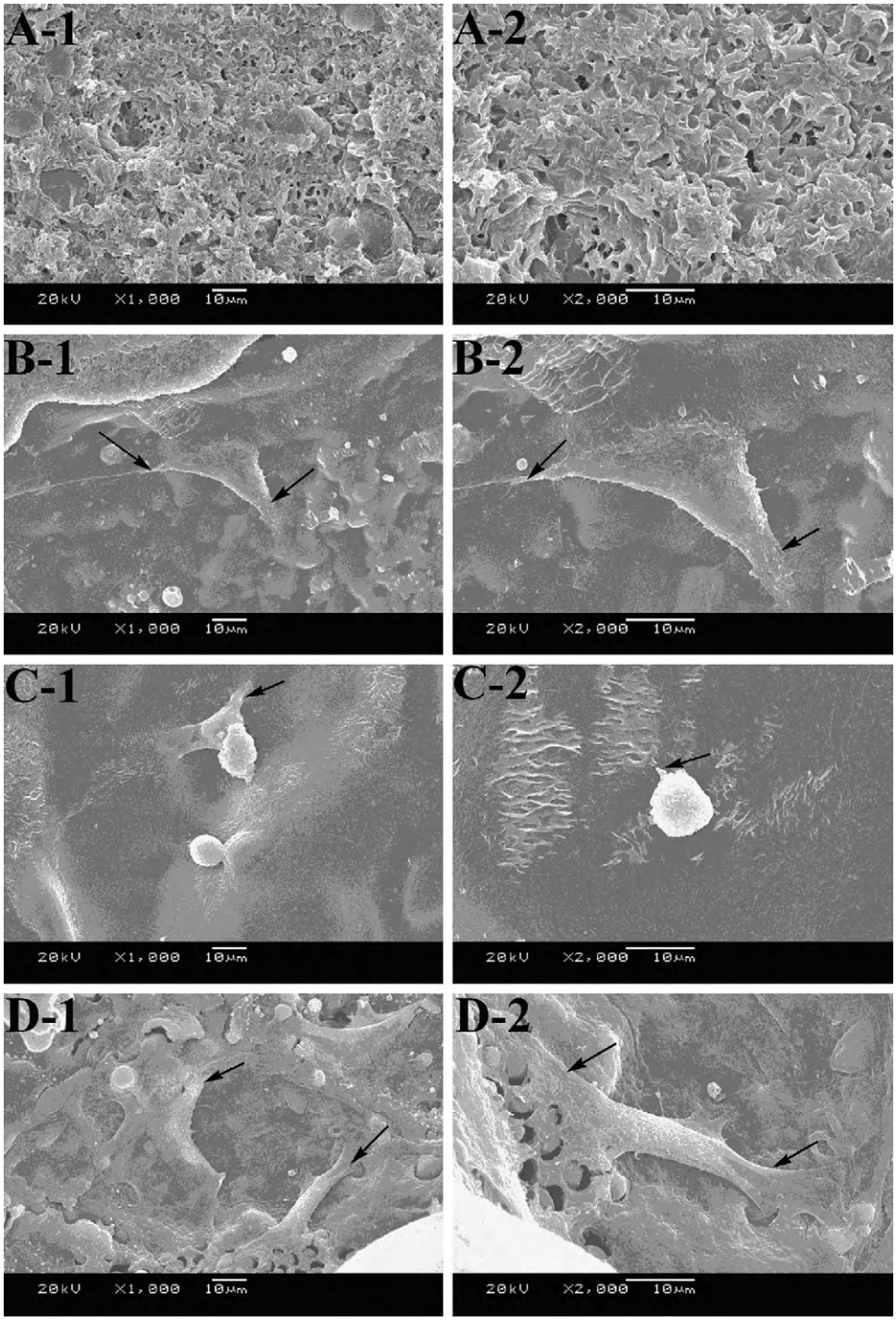


Fig. 8. SEM micrographs of the CEC/chitosan composite films with various CEC contents (10% CEC (A-1, A-2), 30% CEC (B-1, B-2), 50% CEC (C-1, C-2) and 70% CEC (D-1, D-2)) seeded with ROS cells after 24 h incubation. Arrows represent the cells microvillus.

human serum, chitosan is mainly degraded by lysozyme, but not by other enzymes or other depolymerization mechanisms (Freier et al., 2005; Lee et al., 1995; Nordtveit, Varum, & Smidsrod, 1994). As the early report of Pangburn, Trescony, and Heller (1982), lysozyme has a hexameric binding site, and hexasaccharide sequences containing 3–4 or more acetylated units contribute to the initial degradation rate of *N*-acetylated chitosan. As depicted in Fig. 7B, blank chitosan film showed significant enzymatic degradation in the study period with the maximum weight loss at about 22%. And the main weight loss occurred in the first 7 days. After a high initial weight loss, the degradation rate of the samples generally appeared to slow down, which is a result of the loss of appropriate hexasaccharide sequences with progressing degradation (Nordtveit et al., 1994). For the CEC/chitosan composite films, the degradation rate decreased significantly as the CEC content increased from 10% to 70%, yet there was no significant difference in degradation behavior among the films with 30% CEC blending, 50% CEC blending and 70% CEC blending. This might be explained by that the introduction of CEC into chitosan film could interfere the enzyme–substrate binding. Meanwhile, the introduction of CEC was not degraded by lysozyme (Li et al., 2010). However, Freier et al. (2005) suggested that the degree of acetylation (DA) and molecular weight (Mw) of chitosan were the most important factors on the degradation rate of chitosan film. Therefore, various DA and Mw of chitosan might be used to tailor the degradation behavior of composite films suitable for the expected applications.

3.9. *In vitro* cytotoxicity

Previous studies suggested that the PCL/chitosan composite films showed the well biocompatible to the fibroblasts (L929 cells) under the *in vitro* testing conditions (Sarasam & Madihally, 2005). Furthermore, 50% and 70% PCL blends showed better support for mouse embryonic fibroblast spreading and survival relative to individual polymers. In this paper, we selected the ROS cells to evaluate the intrinsic cytotoxicity of CEC/chitosan composite films with the extract fluids for 24 h incubation. Fig. 7C shows the cell viability assessed by the MTT test. According to Fig. 7C, we could find that all samples were non-cytotoxic to the ROS cells after 24 h incubation except that of the 10% CEC composite films, which showed the only 25% of viable cells as incubation at 75% and 100% extractions. This interesting phenomenon was in disagreement with the other reports (Sarasam & Madihally, 2005; Sarasam et al., 2006). Sarasam and Madihally (2005) and Sarasam et al. (2006) reported that all the extract fluids of PCL/chitosan (1/4, 1/1 and 3/4; w/w) films were non-cytotoxic effect on the mouse embryonic fibroblasts. And the reason for this interesting phenomenon needs further study.

3.10. Cell adhesion and morphology study

Fig. 8 shows SEM images of ROS cells grown on the CEC/chitosan composite films after 24 h incubation. The microstructure of CEC/chitosan composite films did not seem to have changed after 24 h incubation except that of the 10% CEC composite films. The roughness of 10% CEC composite films increased greatly compared with that of original films, which seems to be partial degradation during the cell incubation. This obvious morphological change indicated that partial polymer was dissolved into the medium, which might result in the cell death. Therefore, we did not observe any cell attachment on the 10% CEC composite films. Meanwhile, due to the high fraction of chitosan (90%) in the 10% CEC composite film, which showed "gel-like" character also leading to the non-adhesive nature of composite films. Recently, working with chitosan/hexadecenoic acid multi-layer films, Richert et al. (2004) demonstrated that there was a decrease in cell adhesion with an increase in layer number, as the surface became progressively more uniform/smooth; while a rougher film offered an increased surface area for cell attachment (Richert et al., 2004). Cell adhesion increased significantly as the CEC content increasing from 30% to 70%, especially for the 70% CEC composite films. Additionally, the formation of cells microvillus was also observed, which is suggestive of cell activation. In our opinion, this result is quite promising since it demonstrated that the CEC/chitosan composite film possesses an adequate pore structure and roughness suitable for the cell adhesion and proliferation.

4. Conclusion

This paper demonstrated the feasibility to prepare CEC/chitosan composite films by combination with these two polymers in a unique 80% aqueous acetic solution. The prepared composite films showed the well miscibility and interphase interaction as identified by FTIR, XRD and DSC. The well mechanical properties of composite films also make them suitable for the tissue engineering applications. Moreover, the *in vitro* cell test demonstrated that the extract fluids of composite films except that of 10% CEC composite film was non-cytotoxic. Cell adhesion as well as morphology was analyzed by SEM, which indicated that the ROS cells appeared to adhere well and exhibited a normal morphology on the surface of composite films after 24 h incubation. The prepared CEC/chitosan composite films showed promising physico-chemical, mechanical and biological properties and may therefore find applications in tissue engineering of bone.

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References

Azad, A. K., Sermsintham, N., Chandrkrachang, S., & Stevens, W. F. (2004). Chitosan membrane as a wound-healing dressing: Characterization and clinical application. *Journal of Biomedical Materials Research B*, *69*(2), 216–222.

Bello, Y. M., Falabella, A. F., & Eaglstein, W. H. (2001). Tissue-engineered skin: Current status in wound healing. *American Journal of Clinical Dermatology*, *2*(5), 305–313.

Freier, T., Koh, H. S., Kazazian, K., & Shoichet, M. S. (2005). Controlling cell adhesion and degradation of chitosan films by *N*-acetylation. *Biomaterials*, *26*(29), 5872–5878.

García Cruz, D. M., Coutinho, D. F., Mano, J. F., Gómez Ribelles, J. L., & Salmerón Sánchez, M. (2009). Physical interactions in macroporous scaffolds based on poly(ε-caprolactone)/chitosan semi-interpenetrating polymer networks. *Polymer*, *50*(9), 2058–2064.

Gou, M. L., Dai, M., Li, X. Y., Yang, L., Huang, M. J., Wang, Y. S., et al. (2008). Preparation of mannan modified anionic PCL–PEG–PCL nanoparticles at one-step for bFGF antigen delivery to improve humoral immunity. *Colloids and Surfaces B*, *64*(1), 135–139.

Gou, M. L., Qian, Z. Y., Wang, H., Tang, Y. B., Huang, M. J., Kan, B., et al. (2008). Preparation and characterization of magnetic poly(ε-caprolactone)–poly(ethylene glycol)–poly(ε-caprolactone) microspheres. *Journal of Materials Science: Materials in Medicine*, *19*(3), 1033–1041.

Hirano, S., Tsuchida, H., & Nagao, N. (1989). *N*-Acetylation in chitosan and the rate of its enzymic hydrolysis. *Biomaterials*, *10*(8), 574–576.

Huang, M. J., Gou, M. L., Qian, Z. Y., Dai, M., Li, X. Y., Cao, M., et al. (2008). One-step preparation of poly(epsilon-caprolactone)–poly(ethylene glycol)–poly(epsilon-caprolactone) nanoparticles for plasmid DNA delivery. *Journal of Biomedical Materials Research A*, *86*(4), 979–986.

Hutmacher, D. W. (2000). Scaffolds in tissue engineering bone and cartilage. *Biomaterials*, *21*(24), 2529–2543.

Ilium, L. (1998). Chitosan and its use as a pharmaceutical excipient. *Pharmacological Research*, *15*(9), 1326–1331.

Jayakumar, R., Prabaharan, M., Reis, R. L., & Mano, J. F. (2005). Graft copolymerized chitosan—Present status and applications. *Carbohydrate Polymers*, *62*(2), 142–158.

Jiao, Y., Liu, Z., & Zhou, C. (2007). Fabrication and characterization of PLLA–chitosan hybrid scaffolds with improved cell compatibility. *Journal of Biomedical Materials Research A*, *80*(4), 820–825.

Kim, I. Y., Seo, S. J., Moon, H. S., Yoo, M. K., Park, I. Y., Kim, B. C., et al. (2008). Chitosan and its derivatives for tissue engineering applications. *Biotechnology Advances*, *26*(1), 1–21.

Kong, X. Y., Li, X. Y., Wang, X. H., Liu, T. T., Gu, Y. C., Guo, G., et al. (2010). Synthesis and characterization of a novel MPEG–chitosan diblock copolymer and self-assembly of nanoparticles. *Carbohydrate Polymers*, *79*(1), 170–175.

Lee, K. Y., Ha, W. S., & Park, W. H. (1995). Blood compatibility and biodegradability of partially *N*-acylated chitosan derivatives. *Biomaterials*, *16*(16), 1211–1216.

Li, J., Chen, Y. P., Yin, Y., Yao, F., & Yao, K. (2007). Modulation of nano-hydroxyapatite size via formation on chitosan–gelatin network film in situ. *Biomaterials*, *28*(5), 781–790.

Li, X. Y., Kong, X. Y., Shi, S., Gu, Y. C., Yang, L., Guo, G., et al. (2010). Biodegradable MPEG-g-chitosan and methoxy poly(ethylene glycol)-b-poly(ε-caprolactone) composite films: Part 1. Preparation and characterization. *Carbohydrate Polymers*, *79*(2), 429–436.

Liu, C. B., Gong, C. Y., Huang, M. J., Wang, J. W., Pan, Y. F., Zhang, Y. D., et al. (2008). Thermoreversible gel–sol behavior of biodegradable PCL–PEG–PCL triblock copolymer in aqueous solutions. *Journal of Biomedical Materials Research B*, *84*(1), 165–175.

Ma, J., Wang, H., He, B., & Chen, J. (2001). A preliminary in vitro study on the fabrication and tissue engineering applications of a novel chitosan bilayer material as a scaffold of human neofetal dermal fibroblasts. *Biomaterials*, *22*(4), 331–336.

Nettles, D. L., Elder, S. H., & Gilbert, J. A. (2002). Potential use of chitosan as a cell scaffold material for cartilage tissue engineering. *Tissue Engineering Part A*, *8*(6), 1009–1016.

Nordtveit, R. J., Varum, K. M., & Smidsrod, O. (1994). Degradation of fully water-soluble, partially *N*-acetylated chitosans with lysozyme. *Carbohydrate Polymers*, *23*(4), 253–260.

Okamoto, Y., Shibazaki, K., Minami, S., Matsuhashi, A., Tanioka, S., & Shigemasa, Y. (1995). Evaluation of chitin and chitosan on open would healing in dogs. *The Journal of Veterinary Medical Science*, *57*(5), 851–854.

Oliveira, F. C., & Barros-Timmons, A. (2010). Preparation and characterization of chitosan/SiO_2 composite films. *Journal of Nanoscience and Nanotechnology*, *10*(4), 2816–2825.

Oliveira, J. M., Rodrigues, M. T., Silva, S. S., Malafaya, P. B., Gomes, M. E., Viegas, C. A., et al. (2006). Novel hydroxyapatite/chitosan bilayered scaffold for osteochondral tissue-engineering applications: Scaffold design and its performance when seeded with goat bone marrow stromal cells. *Biomaterials*, *27*(36), 6123–6137.

Pangburn, S. H., Trescony, P. V., & Heller, J. (1982). Lysozyme degradation of partially deacetylated chitin, its films and hydrogels. *Biomaterials*, *3*(2), 105–108.

Peng, H., Zhou, S., Guo, T., Li, Y., Li, X., Wang, J., et al. (2008). In vitro degradation and release profiles for electrospun polymeric fibers containing paracetanol. *Colloids and Surfaces B*, *66*(2), 206–212.

Perret, R., & Skoulios, A. (1972). Synthese et caracterisation de copolymers sequences polyoxyethylene/poly(ε-caprolactone). *Makromolekulare Chemie*, *156*(1), 143–156.

Ravi Kumar, M. N. V. (2000). A review of chitin and chitosan applications. *Reactive and Functional Polymers*, *46*(1), 1–27.

Richert, L., Lavalle, P., Payan, E., Shu, X. Z., Prestwich, G. D., Stoltz, J. F., et al. (2004). Layer by layer buildup of polysaccharide films: Physical chemistry and cellular adhesion aspects. *Langmuir*, *20*(2), 448–458.

Rodrigues, L. B., Leite, H. F., Yoshida, M. I., Saliba, J. B., Junior, A. S. C., & Faraco, A. A. G. (2008). In vitro release and characterization of chitosan films as dexamethasone carrier. *International Journal of Pharmaceutics*, *368*, 1–6.

Sarasam, A., & Madihally, S. V. (2005). Characterization of chitosan–polycaprolactone blends for tissue engineering applications. *Biomaterials*, *26*(27), 5500–5508.

Sarasam, A. R., Krishnaswamy, R. K., & Madihally, S. V. (2006). Blending chitosan with polycaprolactone: Effects on physicochemical and antibacterial properties. *Biomacromolecules*, *7*(4), 1131–1138.

Sarasam, A. R., Samli, A. I., Hess, L., Ihnat, M. A., & Madihally, S. V. (2007). Blending chitosan with polycaprolactone: Porous scaffolds and toxicity. *Macromolecular Rapid Communications*, *7*(9), 1160–1167.

Shirosaki, Y., Botelho, C. M., Lopes, M. A., & Santos, J. D. (2009). Synthesis and characterization of chitosan–silicate hydrogel as resorbable vehicle for bonelike bone graft. *Journal of Nanoscience and Nanotechnology*, *9*(6), 3714–3719.

Thanpitcha, T., Sirivat, A., Jamieson, A. M., & Rujiravanit, R. (2006). Preparation and characterization of polyaniline/chitosan blend film. *Carbohydrate Research*, *64*(4), 560–568.

Veleirinho, B., & Delgadillo, I. (2009). Preparation and characterization of electrospun mats made of PET/chitosan hybrid nanofibers. *Journal of Nanoscience and Nanotechnology*, *9*(6), 3798–3804.

Wu, C. S. (2005). A comparison of the structure, thermal properties, and biodegradability of polycaprolactone/chitosan and acrylic acid grafted polycaprolactone/chitosan. *Polymer*, *46*(1), 147–155.

Zhou, S., Deng, X., & Yang, H. (2003). Biodegradable poly(ε-caprolactone)–poly(ethylene glycol) block copolymers: Characterization and their use as drug carriers for a controlled delivery system. *Biomaterials*, *24*(20), 3563–3570.

Zhou, S., Song, B., & Li, X. (2007). In vitro degradation and release profiles for poly-DL-lactide film containing paracetamol. *Journal of Materials Science: Materials in Medicine*, *18*(8), 1623–1626.