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A study on cytocompatible poly(chitosan-*g*-L-lactic acid)

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Abstract

A novel cytocompatible graft copolymer of chitosan and L-lactic acid (CL) was prepared by grafting L-lactic acid onto the amino groups in chitosan without a catalyst. The structures of the CL graft copolymers were characterized by FTIR, ^{13}C-NMR and X-ray measurements. Degree of substitution and side-chain length were evaluated from salicylaldehyde and elemental analysis. The tensile strength and water uptake of the CL copolymers films were investigated as a function of feed ratio of LA/CS. The influence of pH on the swelling behavior of the copolymer films was determined and interpreted. Fibroblast culture was performed to evaluate cell proliferation on the copolymers films. The results showed that the cell growth rate on the copolymers films is faster than chitosan obviously.

Keywords: Chitosan; Poly(L-lactic acid); Fibroblast

1. Introduction

Recently, much attention has been given to utilize chitosan in biomedical applications, for example, as a wound dressing, bandage material, skin grafting template, hemostatic agent, hemodialysis membrane and drug delivery vehicle, etc. [1,2]. Chitosan gels have been applied to conduct the extracellular matrix (ECM) formation in tissue regeneration. The superior biocompatibility of chitosan may primarily be attributed to its structural similarity to glycosaminoglycans in ECM. Chitosan has been reported to stimulate the activity of growth factors. In vitro studies have clarified the contribution of chitosan in wound healing through its activation of fibrogenic mediators such as growth factors. Increased expression of growth factors enhanced fibroblastic activity and promoted fibrous tissue synthesis.

In our previous reports, we have prepared the porous chitosan/gelatin network scaffold via polyelectrolyte complex formation [3], and a hydroxyapatite/chitosan–gelatin network [4] (HA/CS–Gel) composite of similar composition to that of normal human bone was also prepared as a three-dimensional biomimetic scaffold. Shanmugasundaram [5] developed a biodegradable polymer scaffold using collagen and chitosan in the form of an interpenetrating polymeric network and gluaraldehyde was used as a cross-linking agent for the development of a scaffold for in vitro culture of human epidermoid carcinoma cell. Yao [6,7] fabricated asymmetric scaffolds composed of chitosan and gelatin, suggesting that would be suitable for skin tissue engineering goals.

Biodegradable polyesters, such as polylactic acid, polyglycolic acid and their copolymers, have been chosen to serve as scaffolds, for copolymers of lactic and glycolic acid undergoing controllable hydrolytic degradation into natural metabolites, and can be processed into highly efficient scaffolds by a variety of methods [8,9].

Poly(α-hydroxy acids) generate acidic degradation products at the implanted site which evokes undesirable tissue reaction [10,11]. The acid by-product may lead to local disturbance due to poor vascularization in the surrounding tissue. Chitosan may be combined with acid-producing biodegradable polymers, so that local toxicity due to the acid by-products can be alleviated.

In our previous research, poly (D, L-lactic acid) was modified with chitosan [12] and its derivatives [13,14]. Chandy [15] coated the microsphere surface of poly(lactic acid)/poly(caprolactone) blend with chitosan for the

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targeted delivery of antiproliferative agents to treat restenosis. Chitosan was also covalently immobilized onto polylactic acid film surface using the photosensitive heterobifunctional cross-linking reagent [16]. Hudson [17] reviewed the graft copolymerization of chitin/chitosan through radical polymerization. Shen [18] immobilized chitosan on PLLA membrane surface using a grafting-coating method with the goal of improving of cellular interactions. Qu [19,20] has synthesized the copolymer of low molecular chitosan and D,L-lactic acid and investigated its properties as a pH-sensitive hydrogel, anticipating its application in a pH-sensitive controlled-release system. We have reported the synthesis and characterization of an amphoteric pH-sensitive biodegradable poly (chitosan-*g*-(L-lactic-co-citric) acid) hydrogel [21].

Here, a graft copolymer of L-lactic acid onto chitosan was synthesized without using a catalyst to improve the mechanical properties of chitosan, especially its brittleness resulting from its high crystallinity. Meanwhile, because of the physical cross-linking due to the aggregation of the hydrophobic side chains, the generally used chemical cross-linking agent such as glutaraldehyde will be avoided. The structure was characterized and pH-sensitive swelling properties, mechanistic strength of CL graft copolymers were investigated. The proliferation of fibroblasts on the CL copolymer films was discussed in comparison with chitosan cross-linked by glutaraldehyde.

2. Experimental

2.1. Materials

Chitosan (CS) ($\bar{M}_v = 1.5 \times 10^5$, the degree of deacetylation DD = 90%) from Yuhuan Ocean Biochemical Co. was used for the preparation of graft copolymer. The degree of deacetylation (DD = 90%) was determined by IR spectroscopy. L-lactic acid (LA) (88% aqueous solution) was obtained from Purac Biochem and used directly. All other chemicals were analytical pure grade and used as delivered, without further purification.

2.2. Preparation of CL graft copolymer

CS powder was dissolved in the aqueous solution of L-lactic acid. The solution was poured into a frame mould and maintained at 65 °C for 5 h for film formation. Then the film was treated at 80–90 °C under a pressure of 5–10 mmHg for 3 h and under the same pressure for another 2 h to promote dehydration of the CS copolymer salts with formation of the corresponding amide linkages. To remove the unreacted L-lactic acid and oligo(L-lactic acid) (OLLA), the sample was extracted with chloroform and methanol separately in a Soxhlet apparatus for 48 h. The CL film was about 0.07 mm thick.

2.3. Swelling behavior of the CL copolymer

Dried CL graft copolymer films (0.5 × 1.5 cm) were left to swell in a solution of the desired pH (1.74–12.80), and an ionic strength $I = 0.5$ mol/l at 37 °C. Swollen films removed from the solution at regular intervals were dried superficially with filter paper, weighed and replaced in the same bath. The measurements were continued until a constant weight was reached for each sample. The degree of swelling, W is expressed as the amount of absorbed water per gram dry polymer during a definite time interval.

$$W = (W_s - W_o)/W_o \quad (1)$$

Where W_s and W_o are the weights of the samples in the swelled and dry states, respectively.

2.4. Characterization

The Fourier-transform infrared (FTIR) transmission spectra were obtained from the film sample on a BIO-RAD FT3000 spectrometer.

The ^{13}C-NMR spectrum of CL graft copolymer was recorded with a Varian UNITYplus 400NMR spectrometer in solid state.

Wide-angle X-ray diffraction investigation of the copolymer was carried out on film samples at room temperature with nickel-filtered Cu K_α radiation. The scan width was 3–60°, and the step length was 0.02°.

Analysis of the C, H and N content of CL graft copolymers was carried out on PE-2400 elemental analysis (EA) instrument.

The degree of substitution (DS) of CS amino groups in CL graft copolymer was determined by the formation of *N*-salicylidene CS. An accurately weighed sample was immersed for 48 h in 100 ml of 0.02 M salicylaldehyde in a methanol/1% acetic acid aqueous solution (80/20, V/V). After 48 h, a portion of the filtrate was diluted 625 times, and the absorbance at 255 nm was measured to determine the residual concentration of salicylaldehyde.

Measurements on mechanical properties of specimens were performed at room temperature with a DZL-50 tensile machine at a cross-head speed of 10 mm/min. Each value reported is an average of five specimens.

2.5. Cell culture

Primary cultures were grown by the method of Rheinwald and Green [22]. Fibroblasts were isolated from dermis by sequential trypsin and collagenase digestion. Cells were cultured in DMEM containing 10% fetal bovine serum, L-gluramine and penicillin–streptomycin. They were incubated at 37 °C in air containing 5% CO_2. The culture medium was changed every 3 days. At confluence, fibroblasts were harvested and subcultivated in the same medium.

2.6. Culture of fibroblast in CL graft copolymer films

Fibroblasts (1×10^5 cells/cm^2) suspended in the medium as above were seeded on chitosan–gelatin scaffolds in a 24-well culture plate and culture for 2, 3, or 4 weeks with medium changes every other day.

2.7. Proliferation assay

In order to determine the proliferation rate of human fibroblasts within chitosan–gelatin scaffolds, the MTT test was performed according to the methods of Mosmam [23] with minor modifications. Briefly, fibroblasts were seeded onto CL graft copolymer films using capillary action at density of 1×10^5 cells/cm^2 in 24-well tissue culture clusters (Falco). Medium was changed every other day. At time intervals, the films were detached and transferred into new wells and MTT was added to establish cell growth. The absorbance of the solution was measured at 490 nm using an ELISA (Biorad). Blank cultures in the absence of biomaterial were used as controls. Experiments were run in triplicate per sample. All data were expressed as mean ± standard deviation(SD) for $n = 3$.

3. Results and discussion

3.1. Graft copolymerization of chitosan and L-lactic acid

As shown in Scheme 1, the amino groups of chitosan will be protonated and the chitosan amino lactate salt formed as chitosan was dissolved in an L-lactic acid aqueous solution. The dehydration of the salt will occur to form amide groups between the chitosan and L-lactic acid by heating the solution, the polycondensation of L-lactic acid occurring at the same time.

Transparent films were obtained after the reaction between chitosan and L-lactic acid as described in Section 2. All of unreacted L-lactic acid, OLLA, amide and ammonium salt links could exist in the as-prepared product due to the difficulty of removing water from the solid state in the amide forming dehydration step. The percentage of each one will depend on the feed ratio and reaction conditions, e.g. temperature, pressure and the thickness of the film, so on. Chloroform or methanol could dissolve the unreacted monomers and oligomers, so CL copolymer can be separated from as-prepared product after extracting with methanol or chloroform. The mass change before and after extracting was calculated according Eq. (2).

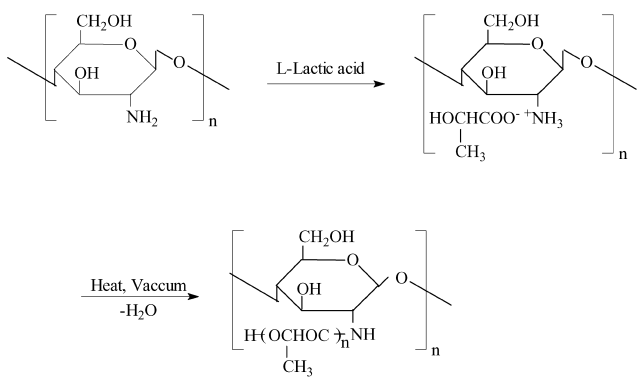
$$\Delta W = (W_0 - W)/W_0 \times 100\% \quad (2)$$

where W_0 is the mass of the as-prepared product, W is the mass after extracting with methanol. Fig. 1 shows the mass change as a function of feed ratio.

It can be seen from Fig. 1 that the mass loss increased along with the rising of the L-lactic acid dosage when LA/CS is lower than 3.0, after that, the mass loss leveled off. During the heating process, the formation of amide and the polycondensation of L-lactic acid took place at the same time. So the content of the oligomer and the unreacted monomer L-lactic acid in the crude product enhanced when raising the amount of L-lactic acid added. As described in the experimented section, both the amidation and polycondensation occurred in solid state, so the specific surface area or the film thickness would have effects on the extent of reaction. As shown in Fig. 1, the thicker the film, and the more difficult the reaction took place that resulting in the increase in unreacted monomer.

3.2. IR analysis of CL copolymer

Fig. 2 shows the IR spectra of chitosan and the copolymer without extracting with methanol or chloroform (CL-0). The IR spectrum of chitosan shows peaks assigned to the saccharine structure at 897 and 1153 cm^{-1} and a strong amino characteristic peak at around 1597 cm^{-1}. The shoulder peak at 1655 cm^{-1} is attributed to the amide I band of N-acylated chitosan. Compared to the IR spectrum of chitosan, the as-prepared CL copolymer (CL-0) has a new peak appearing around 1736 cm^{-1}, corresponding to the ester or carboxylic groups of OLLA existing as freedom or side chain. An obvious shift to a lower wave number in



Scheme 1. Graft copolymerization of chitosan and L-lactic acid.

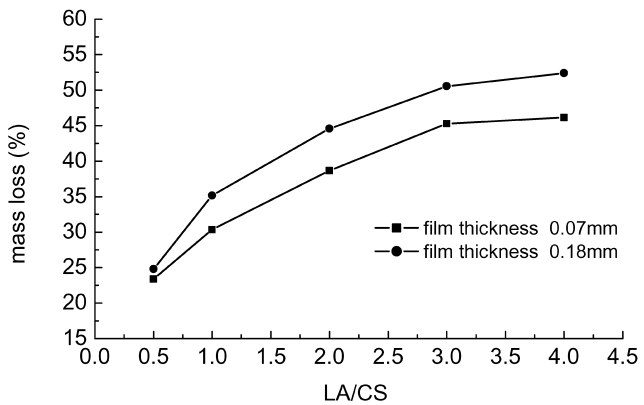


Fig. 1. The mass changes of the as-prepared copolymers after extracting as a function of feed ratio.

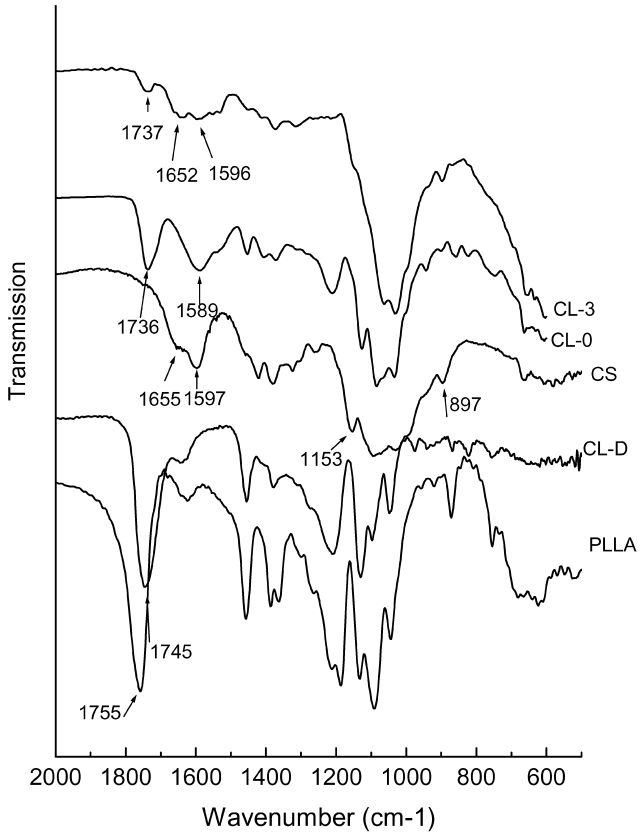


Fig. 2. IR spectra of CL copolymers, chitosan and PLLA.

comparison with PLLA can be observed and is attributed to the formation of hydrogen bonds between the ester groups of OLLA and amino or hydroxyl groups of CS. The new absorption band at around 1589 cm^{-1} is a result of the overlapping of the peaks from the amide I bands and the amino groups of CS with the peaks from the salt links that conjoin CS with OLLA. The shift of the amino groups' absorption from 1597 cm^{-1} of CS to 1589 cm^{-1} of the as-prepared CL copolymer means that OLLA linked with CS both through amide bonding and static electron interaction. No peak corresponding to ether groups from reaction between the hydroxyl groups was found in the IR spectrum of CL-0. So it can be supposed that chemical cross-linking does not occur at the relatively mild graft copolymerization conditions used.

After extracting CL-0 with chloroform and methanol, separately, CL-3 was obtained and its IR spectrum was shown in Fig. 2, too. Compared with CL-0, the absorption at 1737 cm^{-1} attributed to the ester groups of OLLA became weaken, because of the separating of the free oligomer. Compared with CS, the peak at 1652 cm^{-1} assigned to the amide groups became stronger compared with the peak at 1596 cm^{-1} attributed to the amino groups. That demonstrates the formation of amide between CS and L-lactic acid or OLLA.

After the extractive solution of the as-prepared copolymer being concentrated and dried, a light yellow thick liquid CL-D was obtained. Compared the IR spectra of CL-D (Fig. 2) with PLLA, they were nearly the same. So it can be supposed that the extractive was mainly composed of L-lactic acid and free OLLA.

3.3. Degree of substitution and the length of side chain

The results of EA for the extractive CL-D and the graft copolymers were shown in Table 1. By EA and the DS of the chitosan amino groups determined by the formation of *N*-salicylidene, the $\bar{X}_n$ values of the poly(L-lactic acid) side chain were calculated. According to the structure of CL copolymer as shown in Scheme 1 and the deacetylation of CS (90%), the C and N content of the copolymer can be expressed as Eqs. (3) and (4).

$$N = \frac{14}{203 \times 0.1 + 161 \times 0.9 + 72\bar{X}_n \cdot DS} \quad (3)$$

$$C = \frac{(8 \times 0.1 + 6 \times 0.9 + 3\bar{X}_n \cdot DS)12}{203 \times 0.1 + 161 \times 0.9 + 72\bar{X}_n \cdot DS} \quad (4)$$

and $\bar{X}_n$ can be calculated from Eq. (5).

$$\bar{X}_n = \frac{7C/6N - 6.2}{3 \times DS} \quad (5)$$

The DS and the calculated value of $\bar{X}_n$ were also listed in Table 1. Both DS and $\bar{X}_n$ increase with the feed ratio of L-lactic acid to chitosan, approaching a saturation level of about 20–23% and 7–8 for LA/CS $\geq$3. After that, further raising the amount of L-lactic acid mainly resulted in the enhancing of the polycondensation of L-lactic acid and the raising of the oligomer molecular weight. Higher DS can be attained for the thinner films, since the water can evaporate more easily during the dehydration step.

3.4. ^{13}C-NMR analysis of CL graft copolymer

Fig. 3 display the ^{13}C-NMR spectrum of CL graft copolymer. The signal due to C1 carbon, directly attaching to two oxygen atoms, was found around 104.5 ppm which significantly lower magnetic field compared with the signals of the remaining five carbons. The signals at 57.8 (C4), 61.5 (C3) and 68.8 (C5) (ppm) were detected, although the signals became wider due to the different saccharine

Table 1
DS and $\bar{X}_{n,PLLA}$ of CL graft copolymer

No.	LA/CS (wt/wt)	[COOH]/[NH_2] (mole ratio)	DS (%)	C (%)	N (%)	H (%)	$\bar{X}_{n,PLLA}$
CL-D	–	–	–	48.5	0	5.81	
CL-1	0.5	0.89	14.2	45.33	7.97	14.2	1.02
CL-2	1.0	1.79	18.9	45.92	7.11	6.54	2.35
CL-3	2.0	3.58	20.7	46.97	5.18	6.35	7.05
CL-4	3.0	5.37	22.6	47.17	4.82	6.25	7.70
CL-5	4.0	7.16	23.8	47.32	4.57	6.19	8.24

The thickness of the CL graft copolymer film is 0.07 mm.

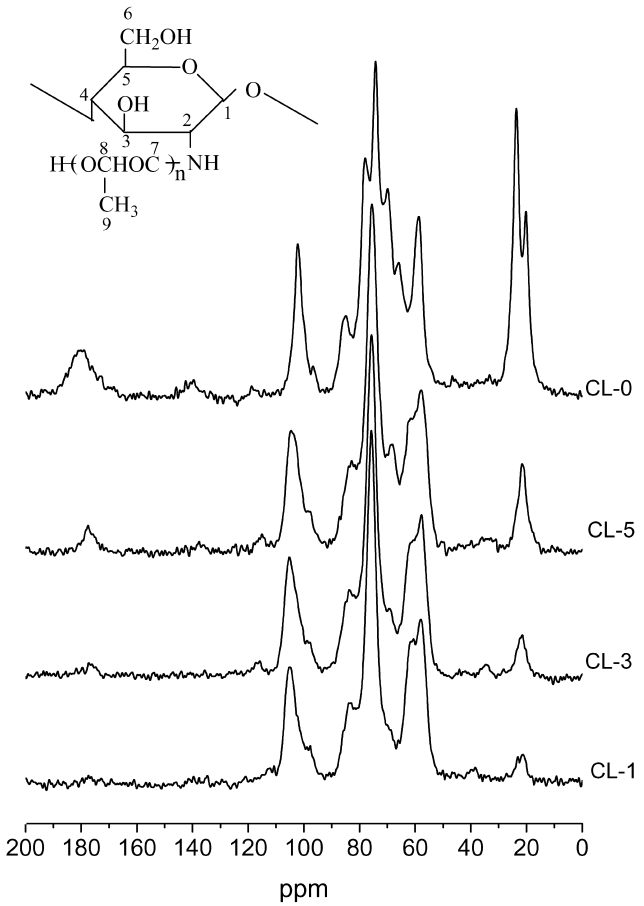


Fig. 3. ^{13}C-NMR spectrum of CL graft copolymers.

structures, such as chitosan, N-acetylated chitosan and CL graft copolymer units. The peaks at 21.4 and 75.7 ppm, were assigned to the methyl and methine carbons of the lactyl unit of the side chain. The wide peak at 176 ppm was attributed to the carbonyl carbon of lactyl. These evidences also support the graft copolymerization of chitosan with L-lactic acid obviously.

Compared the ^{13}C-NMR spectrum of CL-0 and CL-3, the later was obtained after the former was extracted with chloroform and methanol, the peaks attributed to the lactyl moiety of CL-0 were much more stronger than CL-3. Meanwhile, both peaks at 21.4 ppm assigned to C9 and 75.7 ppm assigned to C8 of CL-0 were splited to binary. That also approved the existence of free L-lactic acid and OLLA in CL-0. As for the ^{13}C-NMR spectrum of CL-1, CL-3 and CL-5, peaks at 21.4 and 176 ppm assigned to the OLLA side chain became stronger with the increasing of the feed ratio of LA/CS. That means that the DS of chitosan and the $\bar{X}_n$ of side chain also enhanced.

3.5. Wide angle X-ray diffraction analysis

X-ray diffraction profiles of CS and CL are shown in Fig. 4. Chitosan has an orthorhombic unit cell with $a = 0.824$ nm, $b = 1.039$ nm and $c = 1.648$ nm. The peaks appearing

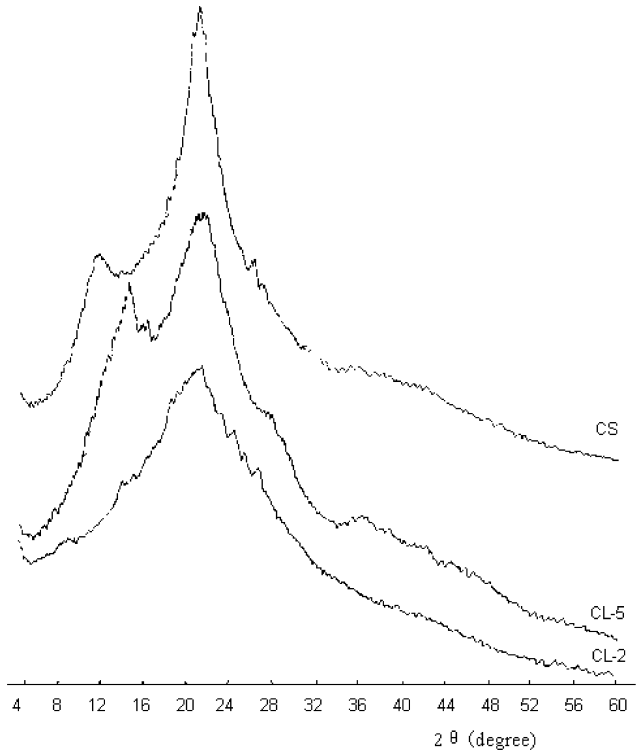


Fig. 4. X-ray diffraction profiles of CS and CL graft copolymers.

at around $2\theta = 10°$ are assigned to (001) and (100), while the peaks around $2\theta = 20°$ are assigned to (020) and (200) [24]. Poly(L-lactic acid) crystallized in a pseudo-orthorhombic unit cell (dimensions $a = 1.07$ nm, $b = 0.595$ nm and $c = 2.78$ nm), which contain two 10^3 helices (α-form), the main peaks in X-ray diffraction profile appeared at 2θ value of 15, 17 and 19 [25].

Compared with chitosan and poly(L-lactic acid), the grafting decreases the intensity at both peaks. When the feed ratio reaches LA/CS = 1, the graft-polymerized samples became almost amorphous. Since L-lactic acid reacts with chitosan in a homogeneous solution, the grafting by PLLA will take place at random along the chain, giving rise to a random copolymer. This will efficiently destroy the regularity of the packing of the original chitosan chains, which results in the formation of almost amorphous copolymer. Otherwise, when the feed ratio LA/CS increased further, the longer PLLA side chains assemble with each other and the peaks in X-ray diffraction profile reappears at 2θ values of 15 and 17 that are attributed to the crystallization of PLLA, the another peak at $2\theta = 19°$ is overlapped with the peak of chitosan at $2\theta = 20°$. That may result to a microscopic phase separation in varying degree.

3.6. Tensile strength of CL graft copolymer

The freshly prepared films of the graft copolymer of chitosan and L-lactic acid (CL) were brittle and transparent. When exposed to air, they absorbed moisture slowly and became soft through plasticization. After extracting with

methanol, it appeared to have better tensile strength and flexibility than those of the initial chitosan and as-prepared sample. Fig. 5 shows the tensile strength of the as-prepared and CL-graft copolymer film as a function of the feed ratio. For the as-prepared film, as the dosage of L-lactic acid increases, the content of the unreacted monomer and the oligomer increases, resulting in the deformation of the strong hydrogen bond between the chitosan chains and the tensile strength of the crude films decrease. As for the CL copolymer films, LA/CS ≤ 2, the increase of the feed ratio of LA/CS enhances the degree of physical cross-linking through hydrophobic side-chain aggregation which would lead to a corresponding decrease of the polymer chain mobility, and the tensile strength increases; when LA/CS ≥ 2, the longer side chains (Table 1) collect together and lead to the microscopic phase separation as approved by X-ray diffraction analysis, accompanied by the decrease of tensile strength.

3.7. Swelling behavior of CL graft copolymer in aqueous solution of various pHs

Fig. 6 shows the effect of the LA/CS feed ratio and pH value on the equilibrium water uptake of the CL films. The ionic strength of the buffers was kept constant ($I = 0.5$ M), since it will largely affect the swellability of the copolymers. When pH < 2, with increase of the buffer pH, the concentration of the charged ionic groups in the films also increase. The swelling of the samples will increase due to enhandement of the osmotic pressure and charge repulsion. While at higher pH, the degree of ionization is reduced due to the deprotonation of the amino units of chitosan and the swelling of the films decreases. In addition, the hydrophobic side-chain aggregation and hydrogen bonds in the copolymers are much stronger, which will also lead to lower swellability of the samples.

As shown in Fig. 6, in the pH 2.37 buffer, the sample with the ratio LA/CS = 0.5 has the highest specific solution content (W) value of 23. The influence of the LA/CS feed ratio on the equilibrium water uptake of the CL copolymers

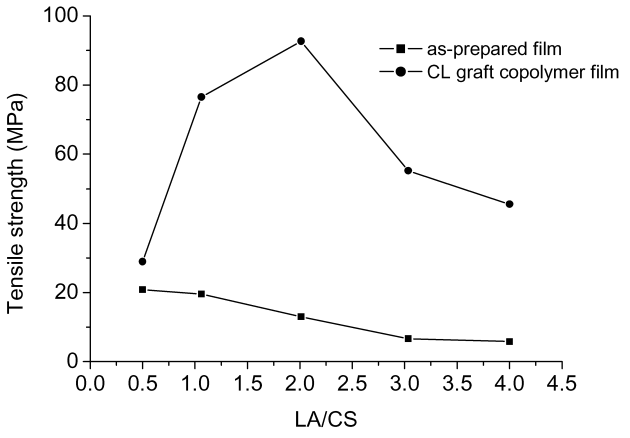


Fig. 5. The tensile strength as a function of feed ratio.

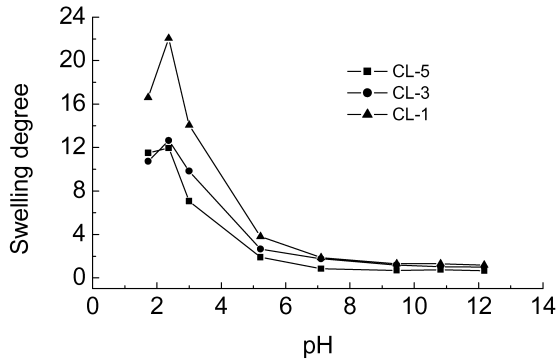


Fig. 6. The effect of the LA/CS feed ratio and pH value on the equilibrium water uptake of the CL films.

is shown in Fig. 7. In the pH 1.73 buffer, the sample with the ratio LA/CS = 0.5 has the highest specific solution content (W) value of 16.5, which decreases abruptly to 12 as LA/CS increases from 0.5 to 1.0. Then, the value levels off as the LA/CS ratio increases from 2 to 4. Because the sample LA/CS = 0.5 has the lowest DS and the shortest side chain length as shown in Table 1, it will form a loosely physical cross-linked copolymer and has more electrostatic repulsion between the protonated amino groups. Thus, the sample LA/CS = 0.5 has the largest swelling among the samples investigated. Moreover, at a ratio of LA/CS = 1.0 where the LA grafts are still short but the DS significantly higher, the water uptake is drastically reduced. After that, the feed ratio of LA/CS only has a small effect on the water-uptake of the CL copolymers.

3.8. Cell proliferation

To further analyze the ability of the composition of the CL copolymer to influence the growth of cultured cells, fibroblasts were cultured on the copolymers films for 11 days and cell activity was assayed with MTT methods at various points along this time course, shown in Fig. 8. Data is the average adsorbance from three different assays. The tissue culture plate was used as controls.

The MTT test is based on mitochondria viability, that is, only functional mitochondria can oxidiae an MTT solution,

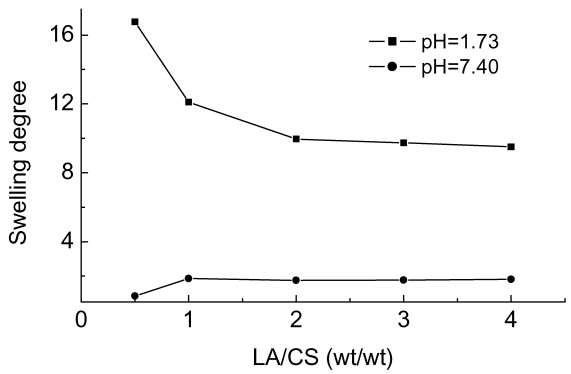


Fig. 7. Influence of feed ratio on equilibrium water uptake of CL copolymers.

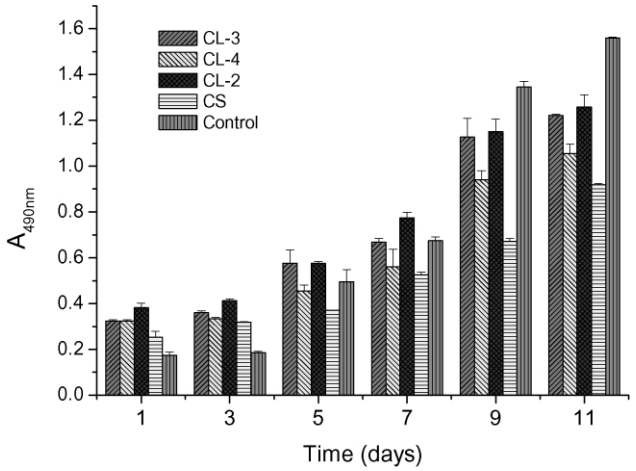


Fig. 8. Proliferation of fibroblasts on CL graft copolymer films with different composition. Error bars represent ± SD for $n = 3$.

giving a typical blue-violet end product. This assay is an indirect method to assay cell growth and proliferation, since the A_{490} values can be correlated to the cell number. In the cell growth profile, the cell growth rate (from 1 to 11 days) in the CL graft copolymer films is related to the feed ratio of L-lactic acid to chitosan. The cell growth rate on the copolymer films is faster than chitosan obviously and decreased when the feed ratio of L-lactic acid to chitosan increases. Compared with chitosan, poly(L-lactic acid) is more hydrophobic. So the balance between hydrophilicity and hydrophobicity of the material had an important effect on the adhesion and immigration of cells on the matrix. The detailed investigation of the reason of the relationship between cell proliferation and the structure of CL graft copolymer is in progress.

4. Conclusions

Through grafting oligo(L-lactic acid) onto the amino groups in chitosan, a novel cytocompatible poly(chitosan-*g*-L-lactic acid) was synthesized and characterized by ^{13}C-NMR, FTIR, X-ray diffraction and EA. The crystallinity of chitosan gradually decreased after grafting, since the side chains substitute the $-NH_2$ groups of chitosan randomly along the chain and destroy the regularity of packing between chitosan chains. The tensile strength of the CL copolymers increased along with the enhancement of feed ratio when LA/CS ≤ 2, after that, the raising of LA/CS resulted in a decrease of tensile strength. In aqueous solutions, the CL graft copolymer could form a pH-sensitive hydrogel due to the aggregation of the hydrophobic side chains. The in vitro fibroblast static cultivation on the films for 11 days showed that the cell growth rate on the copolymers films was faster than chitosan obviously and decreased when the feed ratio of L-lactic acid to chitosan increases. The results suggested that poly(chitosan-*g*-L-lactic acid) as a biomaterials has promising potential in tissue engineering.

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