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Preparation and characterization of polycaprolactone-chitosan composites for tissue engineering applications

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Abstract Highly porous scaffold plays an important role in bone tissue engineering, which becomes a promising alternative approach for bone repair since its emergence. The objective of this work was to blend poly (ε-caprolactone) (PCL) with chitosan (CS) for the purpose of preparation of porous scaffold. A simple unique method was employed under room-temperature condition to blend the two components together without separation of two phases. The reaction leads to formation of sponge-like porous 5, 10, 15 and 20 wt% CS composites. XRD, IR and SEM were used to determine components and morphology of the composites. DSC studies indicated that the miscibility of the two components. And pore volume fractures of composites were determined by a simple method in which a pycnometer was used. The results show that CS is successfully commingled into PCL matrix, and adding CS into PCL will not damage the crystalline structure of PCL. The composite shows no signs of phase separation and presents a unique porous structure under SEM observation. The porosity of composite increased with the increase of the content of CS in the composite. The highest porosity reached to 92% when CS content increased to 20 wt%. The mechanism of formation of this unique porous structure is also discussed.

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Introduction

Tissue engineering can be defined as an interdisciplinary science that applies the principles of engineering and the life sciences toward the development of biological substitutes that restore, maintain, or improve tissue function [1]. Although autograft and allograft are very important in the early stage of hard tissue repair operation, synthetic bone replacement materials are widely used clinical routine [2, 3]. Compared with autograft and allograft, tissue engineering scaffold do not has limitations such as potential infections, supply limitation and additional surgery for harvesting etc. [4]. In the area of bone tissue engineering, scaffold serves as the framework for cell to attach and provide adequate support while occupying specific 3D space for many physical and chemical activities to carry through. Researches over the last two decades have provided us sufficent understandings of inorganic implants such as metallic and ceramic implants [5–8]. Metallic and ceramic implants are not degradable and commonly lack of interconnective porosity. Ideal scaffold should have good porosity to allow tissue ingrowth and have controlled biodegradability to match tissue growth rate thus avoiding stress shielding phenomenon due to modulus-mismatch between bone and implants [9]. Although some attempts have been made to fabricate porous ceramic implants [4, 10], the porosity of reported interconnected structure is no more than 80%. But the ingrowth of bone can only occur if a suitable number of sufficiently large pores are available for the porous structure can provide a template of the ingrowth of bone [11].

Blending two polymers is an effective way to develop new material with combinations of properties not pocessed by individual polymers. Synthetic polymers lack cell-recognition signals and their hydrophobic suface properties make it difficult for cell to adhere on [12]. The wide range

of physical-chemical properties and processabilities of synthetic polymers can integrate with good biocompatibilities and biological interactions of natural polymers by blending synthetic polymers with natural polymers. Polycaprolactone (PCL) is a kind of biodegradable aliphatic polyester with good biocompatibility. And it is an ideal scaffold material for its valuable properties such as nontoxicity for organism, gradual resorption after implantation and good mechanical properties [13]. Chitosan (CS), a poly-2-amino-2-deoxy-β- (1,4)-D-glucopyranose, is obtained from chitin, which is the second most abundant natural polysaccharides. Chitosan has many usages such as sorbent in waste water treatment, wound addressing and drug carrier in pharmaceutical applications because it has been proven to be biodegradable, biocompatible, nonantigenic, nontoxic and biofunctional [12, 14]. Furthermore, the positive charged chitosan is easy to interact with negative charged glycosaminoglycans in the extracellular matrix. Since the properties of PCL and chitosan are complementary, it is possible that blending the two polymers will give composite owning properties of ideal scaffold such as biocompatibility, biodegradability and the ability to promote tissue development [15]. Several studies have been conducted to make PCL-CS composite. Sarasam et al. prepared PCL-CS composite membranes and porous scaffolds in a unique solution of acetic acid [16]. These membranes, however, showed no signs of interconnective porous structure, which is important for the ingrowth of bone tissue. The porous scaffolds were prepared by using freeze-drying method. However, these scaffolds lacked structure integrity and did not form cylindrical structures mimicking the shape of mold.

PCL dissolves in organic solvents such as choloform and tetrahydrofuran. Chitosan, however, is not soluable in most organic solvents. In this study, chitosan and PCL were blended in a common solution of glacial acetic acid and water. Since glacial acetic acid with drops of water is a good common solvent for PCL and CS. The hypothesis is that PCL and CS will precipitate out and form porous scaffold with good miscibility at the same time that NaOH solution is added into the PCL-CS blend system. Different chitosan content composites were prepared and analyzed for in vitro degradation properties, porosities, thermal properties. Results proved the miscibility between PCL and chitosan using Nishi–Wang equation [16–18].

Materials and method

About 250 kD, 80-mesh chitosan (~85% deacetylated powder purchased from Haidebei bioengineering Co. Ltd., Jinan, China) and 70 kD PCL (purchased from Daicel chemical Clo. Ltd., Japan) were used without further purification. All the other chemicals used were of analytical grade.

Formation of solution blends

To prepare 10 wt% PCL solution, PCL was dissolved in glacial acetic acid and kept stirring for 30 min at room temperature. Then certain amount of chitosan powder was put into the solution with 0.5 g water dripped into system afterwards. The system kept stirring for hours until it became pellucid. Four systems of blends, coded as CS05/PCL, CS10/PCL, CS15/PCL and CS20/PCL, with CS contents of 5, 10, 15, 20 wt % by mass were prepared respectively by this method. The ratio of CS/PCL in each system was listed in Table 1. And no CS added system (CS00/PCL) was prepared as control group.

Formation of foams

To prepare porous scaffolds, 50 ml 20 wt% NaOH solution was dripped into the blends of PCL and CS. The amount of NaOH should be enough to neutralize acetic acid in the blends. Phase separation occurred during this process and foam like porous scaffold formed gradually. Foams as-prepared were put into vaccume system and washed by distilled water.

Structure, morphology and thermal property analysis

The crystal structure of prepared composites was investigated by XRD analysis. The prepared foams were cut into slices and then pressed into films, which were then characeterized by Philips X'pert MPD diffractometer using Cu

Table 1 The ratio of CS/PCL in each system

	CS/PCL	CS(g)	PCL(g)	HAc(g)	NaOH(g)
CS00/PCL	00/100	0.00	1.5	13.5	10
CS05/PCL	05/95	0.08	1.5	13.5	10
CS10/PCL	10/90	0.08	1.5	13.5	10
CS15/PCL	15/85	0.08	1.5	13.5	10
CS20/PCL	20/80	0.08	1.5	13.5	10

$K\alpha$ gennerated at 40 KV and 40 mA. The thin film samples were scanned from 10° to 90° with a step size of 0.02° and a count rate of 3.0°/min. Presence of CS in composite was determined by IR analysis. A nicolet Avatar 360 spectrometer, with KBr pastille, was used for FTIR characterization. The analysis range was from wave number 4000–675 cm^{-1}.

The morphology of prepared foams was investigated using a Philips XL30 environmental electron microscopy. Thermal properties of prepared foams were tested on a Mettler Toledo 822e to assess the miscibilities of the blends. Nitrogen flowed as purge gas at the rate of 20 ml/min. Five to ten mg samples of scaffolds were sealed in aluminum pans and heated from 30 to 100°C at the rate of 10°C/min. The melting temperature of PCL was taken from the endothermic peak analyzed by STARe software.

In vitro biodegradation test

Physiology salt solution was prepared by dissolving sodium chloride in de-ionized water to make 0.9 wt% sodium chloride solution. Four groups of prepared foams, CS05/PCL, CS10/PCL, CS15/PCL, CS20/PCL, were soaked in physiology salt solution at 37°C. Each group contained three slices of sample. The pH changes of solutions were taken every 8 days.

Porosity test

The traditional way of measuring scaffold porosity is involved with mercury [19, 20]. But water and other liquids are also employed to calculate porosity [20, 21]. To get the porosity, different samples were washed and dried at 45°C for 1 day. After weighting (Ws), the sample was put into a pycnometer filled with ethanol, the weight of pycnometer and ethanol was taken as W1. Then put the pycnometer into a vacuum container to extract the air out of the sample thus pushing ethanol into the space formerly occupied by air bubble. During the vacuum process, the fluid level in the pycnometer fell down. Took out and Filled up the pycnometer then took the whole weight (W2). After then, took out the sample and dripped the suface ethanol back into the pycnometer to get the weight of the rest ethanol and the pycnometer (W3). The porosity can be calculate from the following equations [22], where ρ is the density of ethanol:

$$\text{Scaffold volume: } Vs = (W1 - W2 + Ws)/\rho \quad (1)$$

$$\text{Pores volume : } Vp = (W2 - W3 - Ws)/\rho \quad (2)$$

$$\text{Porosity : } \varepsilon = Vp/(Vp + Vs) = (W2 - W3 - Ws)/(W1 - W3) \quad (3)$$

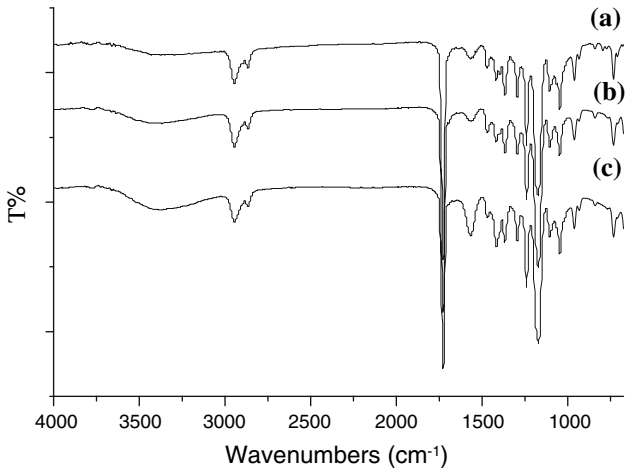

Fig. 1 FTIR spectra of composite foams (**a**) CS00/PCL; (**b**) CS10/PCL; (**c**) CS20/PCL

Results

Characterization of foam like scaffolds

As can be seen from Fig. 1, the FTIR spectra of CS20/PCL (Fig. 1c) shows more intense peaks between 3200 and 3700 cm^{-1} than that of CS00/PCL (Fig. 1a). In Fig. 1a, the peaks between 3000 and 3600 cm^{-1} is characteristic of O–H sretching aborbance band. The more intense peaks at 3200–3700 cm^{-1} in Fig. 1b, c are caused by N–H group of chitosan [23]. The higher absorption intensity of 3200–3700 cm^{-1} in curve CS20/PCL indicates that there is more CS in CS20/PCL compared with CS10/PCL.

The crystal structure of prepared foams can be seen from Fig. 2. Adding CS into PCL matrix makes no changes to crystal structure of PCL. While compared with the miscibility test below, it can be learned that there were weak interactions between PCL and CS caused by blending of the two polymers. Because this kind of weak interactions made little changes to the structure of PCL microcrystallite, the existence of CS caused little changes of XRD patterns of PCL. But the existence of CS may make it difficult for PCL to form lamellar crystal. The change of lamellar structure did not make any difference in XRD patterns, but the thickness decrease of lamellar crystal should cause a small decrease of melting point, which is compatible with DSC result below.

The miscibility between two polymers can be tested by monitoring the melting point depression of the crystalline polymer. The melting point of PCL is around 65°C while CS undergoes thermal degradation around 290°C [24]. Thus, the change of melting point of PCL was used to obtain information about miscibility of blend. The interaction parameter χ_{12} calculated from change of melting

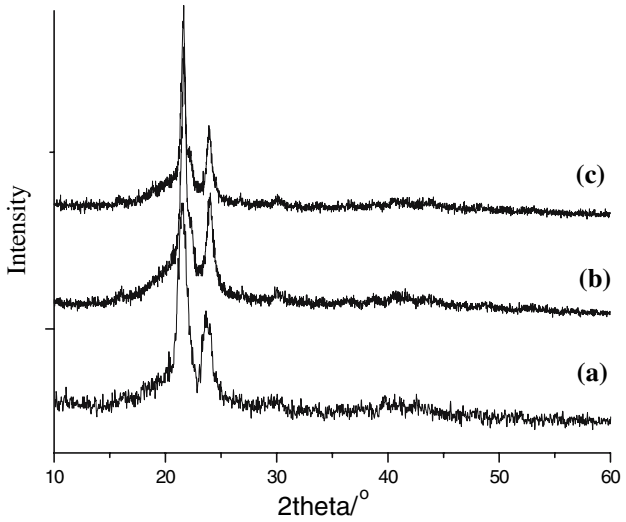

Fig. 2 XRD patterns of composite foams (**a**) CS00/PCL; (**b**) CS10/PCL; (**c**) CS20/PCL

point is widely used for miscibility assessment of polymer blend. A negative or near zero value indicates good miscibility between polymer blend.

The thermograms (Fig. 3) shows a decrease of PCL melting point with increase in CS content. According to Nishi–Wang equation [16–18], the interaction parameter was determined by the following equation:

$$\frac{1}{T_m^o} - \frac{1}{T_{m2}^o} = \frac{-RV_{2u}}{\Delta H_{2u} V_{1u}} \chi_{12} (1 - \psi_2)^2 \quad (4)$$

where T_m^o is the melting point of PCL in the blend, T_{m2}^o is the melting point of pure PCL, R is universal gas constant (8.314 J/mol K), ΔH_{2u} is the heat fusion of pure PCL (15513.88 J/mol), V_{2u} and V_{1u} are molar volumes of the

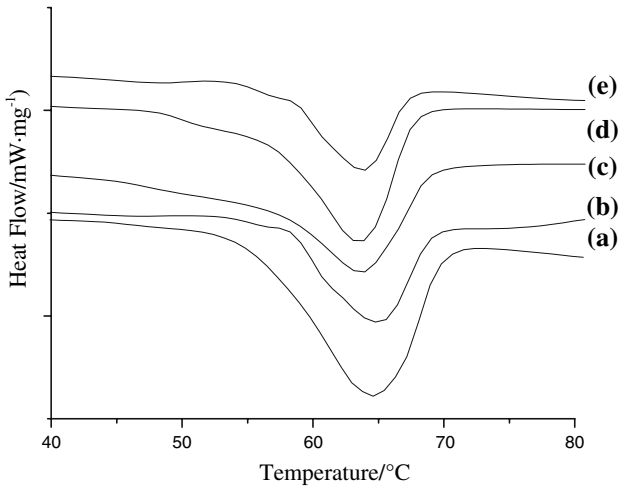

Fig. 3 DSC thermograms of different samples (**a**) CS00/PCL, (**b**) CS05/PCL, (**c**) CS10/PCL, (**d**) CS15/PCL, (**e**) CS20/PCL

Table 2 Melting point depression and interaction parameter values

CS content	Blend melting point/°C	χ_{12}
0.05	64.22	–22.4
0.10	63.62	–20.9
0.15	63.40	–11.8
0.20	63.20	–7.9

repeating units of the polymers (V_{2u} is 99.65 cm^3/mol, V_{2u} is 1546.82 cm^3/mol). ψ_2 is the volume fractions of PCL in the blend. The interaction parameter χ_{12} is negative according to Eq. 4. The results (Table 2) shows that all values of interaction parameter were negative indicating good miscibility between PCL and CS.

Figure 4 shows different fractured surface morphology of samples with addition of 0, 5, 10, 15, 20 wt% chitosan. Seen from Fig. 4a, f, there exist many holes with diameter of 10–30 μm which seem like to be a result of disorder stacking of PCL fibers when PCL precipitated from solution. Obviously different from this kind of random structure, the structure of composite foam owns a honeycomb-like structure. For example, it is evident that foam with addition 5 wt% CS is composed of 3D continuous fiber stretching in space like the root of tree, shown in Fig. 4b. The inner structure of the 3D fiber is very like the structure of honeycomb, shown in Fig. 4g. In other words, the composite foam owns a porous structure composed of 3D fibers, which have an inner structure of honeycomb-like type. From analysis of Fig. 4b, the hole size distribution of this honeycomb structure was measured by imageJ software, as shown in Fig. 5. The average hole area is 244 μm^2.

Porosity studies

Figure 6 shows the influence of CS content on porosity value. It can be seen that the porosity increases with the increase of CS content in the blend.

In vitro studies

After incubation at 37°C for 32 days, the pH value of all the four groups of physiology salt solution increased from 6.7 to more than 8.0, shown in Fig. 7. Because degradation of PCL will lower the pH value of solution, this pH increasement should be caused by degradation of CS.

Discussion

This study was focused on fabrication of PCL/CS composite scaffolds with high porosity and better bioactivity

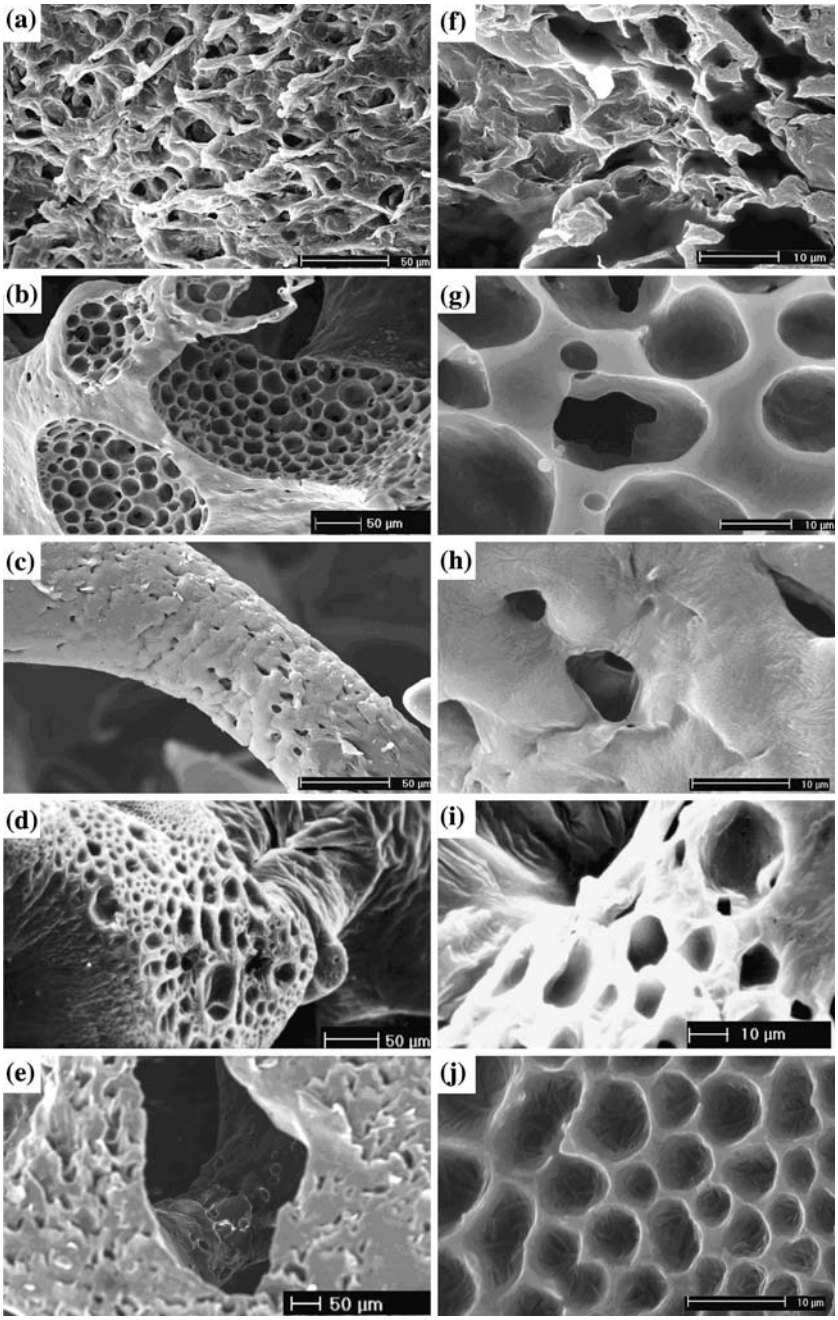

Fig. 4 SEM of the samples (**a**, **f**) CS00/PCL; (**b**, **g**) CS05/PCL; (**c**, **h**) CS10/PCL; (**d**, **i**) CS15/PCL; (**e**, **j**) CS20/PCL

compared with single component. A method was adopted to cast composite foam in blend solution, in which solution (acetic acid) reacted with non-solution (NaOH solution). By this method, CS was successfully commingled into PCL matrix. Seen from Fig. 1, composite containing CS exhibited intense peaks at 3200–3700 cm^{-1} caused by N–H group of CS. The miscibility between CS and PCL was affirmed by results of thermal analysis using Nishi–Wang equation (seen from Table 2), indicating CS was uniformly distributed in PCL. And adding CS did not change the crystal structure of PCL matrix.

The ideal scaffold should be able to promote tissue ingrowth and cell adhesion. The structure with high porosity is expected to contribute to promotion of bone tissue ingrowth. Adding positive charged CS will promote the composite bioactivity since it can interact with negative charged glycosaminoglycans in the extracellular matrix. The foam like scaffold, composed of 3D fibers, which have a honeycomb-like inner sturcture, was formed due to phase separation occurred in the blend solution. Many theories concerning phase separation in porous material have been brought out [25] while few models can match our experi-

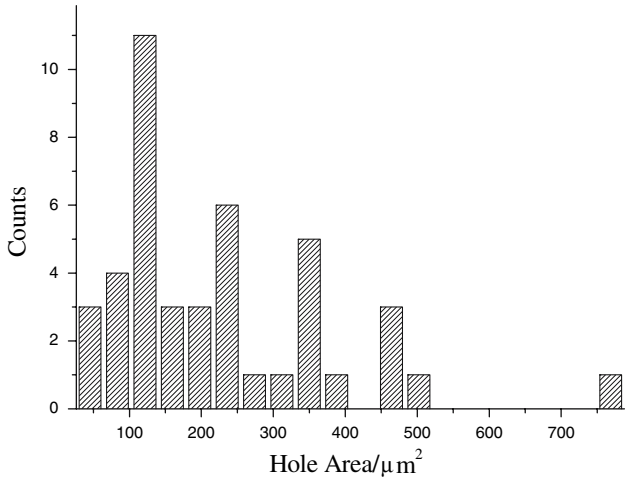

Fig. 5 Hole size distribution of Fig. 4b

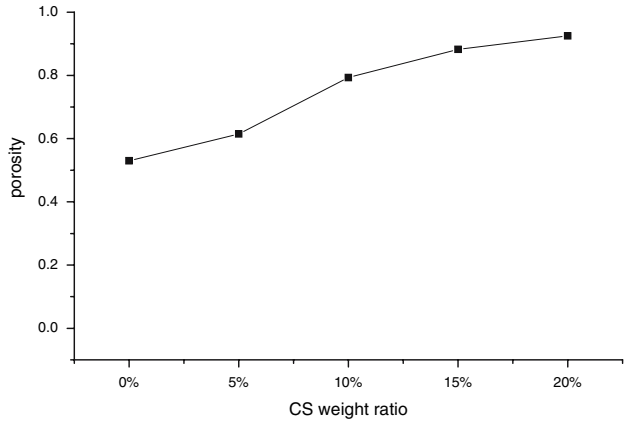

Fig. 6 Relation between porosity and CS content in the blends

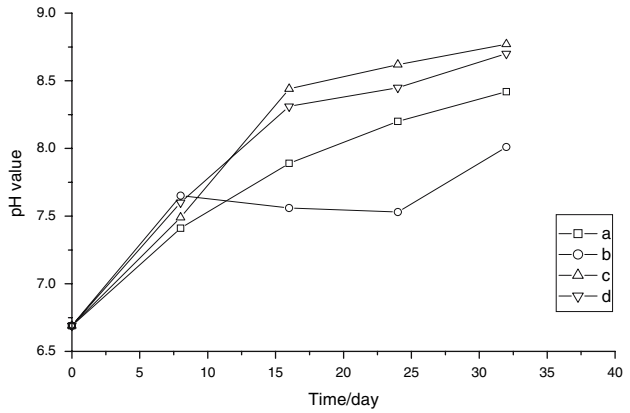

Fig. 7 In vitro pH change test (**a**) CS05/PCL; (**b**) CS10/PCL; (**c**) CS15/PCL; (**d**) CS20/PCL

ment results directly. According to Tanaka's theory [26, 27], a new model, the 'viscoelastic model', is needed to describe the phase separation behaviour of a dynamically asymmetric mixture, which is composed of slow and fast components. In this theory, the dynamic asymmetry can be induced by large size difference and difference in glass-transition, both of which exist between CS and PCL. The fast component has a short relaxation time, which is related with lower glass transition temperature (Tg) compared with that of slow component. In this model, CS is a slow component with a Tg of 140–150°C and PCL is a fast component whose Tg is about –60°C. The deformation rate varies during the phase separation. The deformation rate is not constant and will increase during phase separation. When the slow component's relaxation rate can not catch up with the deformation speed, the slow component behaves as an elastic body leading to the formation of sponge like structure. So, the difference between deformation rate and relaxation rate leads to the formation of spongelike structure and volume shrinkage of slow component. From Tanaka's 3D simulation [27], we can see that our experiment results (root like fibers shown in Fig. 4) share some morphology similarities with Tanaka's 3D simulation (please refer to Fig. 24 of [27] at page R254).

The following is the detailed analysis of the formation of honeycomb-like structure inside of the fibers. First, CS could dissolve in the diluted acid because the free amino group of CS absorbed a hydrogen ion and formed CS-NH_3^+. So the molecular chains of CS tended to keep away from each other and intermingle with PCL molecular chains. During this process, CS and PCL underwent the process of swelling and dissolution. The precipitating pH of CS was about 6.0 and the precipation of PCL only needed water. When NaOH solution was driped in CS/PCL blend system, PCL quickly precipated and formed a membrane at phase interface between water solution and acetic acid solution. The acetic acid solution sealed in this membrane was temporarily protected from the outside water and sodium hydroxide. As time passed by, more and more NaOH solution entered into the acetic solution system and some water and OH^- began to penetrate through the PCL membrane (The permeation of PCL membrane has been proven by Lin's work [28]) while PCL was forming crystalizing layer and CS molecules was solidifying with PCL [4] at the same time.

When the above process was over, partly connected holes were left due to volume shrinking effect in the process of precipitation. Because CS swelled far more than PCL, the former accounted for most of the superabundance volume. Considering the fact that no inner holes were detected in the control group compared with CS05/PCL group under ESEM, we can safely draw the conclusion that the holes were occupied by CS before precipitation. And our porosity study shows that the porosity can be changed by altering CS content in the composite. This can be explained from the above explanation. When NaOH solution

penetrated through PCL membrane, many partly connected holes were left due to volume shrinkage effect in the process of precipitation. Because CS swelled far more than PCL, the former accounted for most of the superabundance volume. The more CS contained in the blend, the more volume occupied by CS became holes after CS was precipitated. So, CS acted as a porogen as well as component of blend. And this relation affirms our hypothesis about the formation of the foams.

Conclusion

In this study, scaffold with interconnective porous structure was prepared successfully by blending chitosan and polycaprolactone in acetic acid solution. The presence of chitosan in composite was proved by IR results. And through XRD analysis, it was clear that adding chitosan into would not change the crystal structure of caprolactone. The melting point of composite foams decreased when chitosan was increased. The miscibility of prepared composite was proved through DSC analysis. The composite foam had a unique honeycomb-like stucture and a high volume porosity ranging from 80% to 90%. The porosity increased with the increase of CS in the blend because the superabundant volumes in the blend solution were mainly determined by CS. The unique sturcture of sponge-like scaffold was a result of viscoelastic phase separation while further studies are still needed to get the detail mechanism of the formation of this porous structure.

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