Biocompatibility of Poly(ε-caprolactone) Scaffold Modified by Chitosan—The Fibroblasts Proliferation in vitro

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ABSTRACT: In this study, the surface of poly(ε-caprolactone) (PCL) scaffold was modified by chitosan (CS) in order to enhance its cell affinity and biocompatibility. It is demonstrated by scanning electronic microscopy (SEM) that when 0.5–2.0 wt% chitosan solutions are used to modify the PCL scaffold, the amount of adhesion of the fibroblasts on the chitosan-modified PCL scaffolds dramatically increase when compared to the control after 7 days cell culture. The results indicate that the chitosan-modified PCL scaffolds are more favorable for cell proliferation by improving the scaffold biocompatibility. The improvement may be helpful for the extensive applications of PCL scaffold in heart valve and blood vessel tissue engineering.

KEY WORDS: PCL, chitosan, porous scaffold, modification, human fetal lung fibroblast (HFL-1).

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INTRODUCTION

In tissue engineering, the scaffold serves as a three-dimensional (3D) template for cell adhesion, proliferation and formation of an extracellular matrix (ECM), as well as a carrier of the growth factors or other biomolecular signals [1]. An ideal scaffold for tissue engineering should have considerable mechanical properties, suitable biodegradability and, most importantly, good biocompatibility [2–4]. In particular, the surface properties of the materials will determine the interactions between the cells and the materials and, in consequence, affect the cell adhesion [5].

Poly(ε-caprolactone) (PCL), which is semicrystalline and biodegradable [6], is a commonly used material for tissue engineering and has been widely used in the medical field for the past 30 years [7–9]. Compared with other commonly used scaffold materials such as polylactide acid (PLA) and polyglycolic acid (PGA), PCL has a good mechanical property and can be easily processed [10]. It has been regarded as a soft and hard tissue-compatible biodegradable material [11] and has FDA approval. However, due to its intrinsic hydrophobic properties and lack of bioactive functional groups, PCL is not very favorable for cell growth, which restricts its applications for tissue engineering [12].

Many methods have been developed to modify the surface of the scaffold to improve its biocompatibility [13–18]. Among these methods, the modification of synthetic materials with natural products is an easy and efficient way to prepare the hybrid scaffold with combined advantages of both the components. Chitosan (CS), as a semicrystalline natural polymer, has good hydrophilicity and biocompatibility [19]. The potential of chitosan as a biomaterial stems from its cationic nature and high charge density in solution. The N-acetylglucosamine moiety in chitosan is structurally similar to glycosaminoglycan (GAGs), which holds the specific interactions with growth factors, receptors, and adhesion proteins. Thus, the analogous structure in chitosan may also have the same bioactivity [20]. In addition, there are many reactive amino and carboxyl groups on the molecules of chitosan that can be chemically modified by introducing new functional groups. Therefore, interest has been focused on the preparation of such a chitosan-modified hybrid scaffold with good mechanical properties and degradability [21–24]. Many researchers [25–34] have examined the tissue response to the various chitosan-based implants. The cell growth on these scaffolds suggested that the introduction of chitosan could significantly improve the biocompatibility of those synthetic materials such as PLA and PGA.
Moreover, it is found that these complex materials evoke a minimal foreign body reaction.

In the present work, we attempt to study the biocompatibility of a chitosan-modified PCL scaffold in vitro for human fetal lung fibroblasts. Because a stable combination of two incompatible polymers can be achieved by a porous structure and a hydrogen bonding between the two polymers, a three-dimensional porous structure of PCL was fabricated. Importantly, the chitosan used in this work can form hydrogen bonding interactions with PCL between the hydroxyl group of chitosan and the ester group of PCL, therefore resulting in the firm adsorption of chitosan to PCL. All the results would be helpful to develop the applicability of PCL for more extensive tissue engineering.

MATERIALS AND METHODS

Materials

Poly(ε-caprolactone) with a molecular weight of 80,000 (Mn) was purchased from Aldrich, and chitosan with an average molecular weight of 30,000 and 65% deacetylation were purchased from Dalian Chitin Limited Company, China. The human fetal lung fibroblasts (HFL-1) were purchased from American Type Culture Collection (ATCC, Rockville, MA, USA). The culture media was Dulbecco’s Modified Eagle’s Medium (DMEM, Gibco, Carlsbad, CA, USA) supplemented with 10% (V/V) heat inactivated newborn calf serum (NCS, Gibco), 100 U/mL penicillin and 100 μg/mL streptomycin (Gibco). Other reagents used included M199 RPMI 1640 culture medium, PBS buffer, were also purchased from Gibco; 0.25% trypsinase and 3-(4,5-dimethylthiazol-2-yl)-diphenyltetrazolium (MTT) were purchased from Sigma Company.

Scaffold Fabrication and Characterization

Preparation of PCL Scaffold

Three-dimensional porous PCL scaffolds were prepared by the particle-leaching technique using sodium chloride particles as the porogen. Nine grams sieved NaCl particles with different diameters ranging from 400 to 120 mesh (Table 1) were added to a 10 mL 10% (w/v) PCL dichloromethane solution. The solution was stirred vigorously to disperse the particles evenly, and cast equally in three circular glass dishes with 60 mm diameter. The solvent was allowed to evaporate
for 24 h in a fume hood, achieving the PCL–salt composite scaffolds. After 10 h vacuum drying to eliminate the residual dichloromethane, the scaffolds were immersed into deionized water for 3 days to leach out the salts, with the deionized water being replaced every 6 h. A three-dimensional porous scaffold was formed, then dried and weighed before further use. The pore size of the scaffold can be expressed by $d = (l \times h)^{0.5}$ (1), where $l$ represents the length of the pore and $h$ represents the width of the pore observed through a scanning electron microscope (SEM, Philips XL30). An average pore size was determined by randomly selecting 10 pores. As observed earlier [36], the scaffold pore size is determined dominantly by the particle size. For the NaCl porogen with particle sizes of 40, 50, 75, 100 and 125 µm, we found the obtained scaffold pore size is within the range of 35–50, 50–75, 75–100, 100–125, and >125 µm, respectively.

**Preparation of Chitosan-modified PCL Scaffold**

First, 3.0 g chitosan was dissolved in 100 mL 2.0% (v/v) acetic acid solution, then 0.5, 1.0, 1.5, 2.0, and 2.5 (w/v) solutions were obtained by diluting the above solution using 2% acetic acid solution.

The porous PCL scaffold prepared earlier was immersed in the different concentrations of chitosan solutions overnight under 0.05 MPa vacuum pressure to allow the chitosan to efficiently adsorb onto the scaffold thus forming CS-coated scaffolds. These CS-coated scaffolds were frozen in liquid nitrogen for 5 min, followed by lyophilization overnight under a vacuum of 0.1 MPa at –50°C. The freeze-dried samples were immersed into 5% NaOH solution to neutralize the remaining acetic acid in the chitosan, and then washed with deionized water until the scaffolds were at a neutral pH.

**Morphology of a Scaffold**

The morphology of the surface and the cross section of both the CS-modified porous PCL scaffolds and the nonmodified, original one (control), were observed by SEM at an accelerating voltage of 15–20 kV.

**Table 1. The relation between used NaCl particle size and the pore size of the obtained scaffold.**

<table>
<thead>
<tr>
<th>NaCl particle size (mesh)</th>
<th>120</th>
<th>150</th>
<th>200</th>
<th>300</th>
<th>400</th>
</tr>
</thead>
<tbody>
<tr>
<td>Scaffold pore size (µm)</td>
<td>125</td>
<td>100</td>
<td>75</td>
<td>50</td>
<td>40</td>
</tr>
</tbody>
</table>

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Adsorbed CS Content and Stability in the Modified Scaffolds

The content of adsorbed CS in the scaffold was calculated with the following expression:

$$\text{CS\%} = \frac{W_m - W_{PCL}}{W_m} \times 100\%$$

where $W_{PCL}$ is the weight of the PCL scaffold before CS coating; $W_m$ is the weight of the modified-scaffold.

The stability of the adsorbed CS in the modified matrices was evaluated in physiological-like conditions after the modified scaffolds were immersed into phosphate buffered saline (PBS, pH = 7.4) solution and incubated at 37°C up to 7 days. The scaffolds were taken out from the PBS, washed with distilled water, vacuum-dried, and weighed. The ratio of CS retained ($R_t$) was defined as the following expression to evaluate the adsorbed CS stability in the scaffold:

$$R_t\% = \frac{W_t - W_{PCL}}{W_0 - W_{PCL}} \times 100\%$$

$W_{PCL}$ represents the weight of the PCL scaffold before CS was coated. $W_0$ and $W_t$ represent the weights of the CS-modified scaffolds before and after immersion into PBS solution for a given time, respectively. This definition implies that the initial CS-retained ratio $R_0$ before incubation was 100%. $R_t$ was measured at various immersion times up to 7 days.

Cell Culture on the Scaffolds and Characterization

Preparation of Cells

After fibroblasts in the culture flask grew to be confluent, they were digested with 1 mL 0.25% trypsinase for 2 min to create a single cell suspension, then 3 mL of culture medium was added to stop the digestion. Cell numbers were counted using a hemocytometer (Shanghai Yuejin Medical Instrument Company, China). The cells were then diluted to a concentration of $7.5 \times 10^5$ cells/mL for further use.

Cell Cultured on the Scaffolds

Both CS-modified scaffolds and control ones were cut into small circular pieces of diameter 15 mm and sterilized with 75% (v/v) ethanol solution overnight. The scaffolds were additionally sterilized with
ultraviolet light for 2h. The sterilized scaffolds were preincubated in a pH = 7.4 PBS solution to replace the ethanol remaining in the samples and then were transferred to a new sterile 24-well cell culture plate (Costar®). One hundred microliter cell suspension was dripped into each well. After the cells were incubated in a humidified incubator (5% CO₂, 37°C) for 1h, an additional 1mL culture medium was added into each well, with the medium being replenished every 2 days.

**MTT Assay**

The cell numbers of viable fibroblasts on the scaffolds were quantitatively assessed with 3-(4,5-dimethylthiazol-2-yl)-diphenyltetrazolium bromide (MTT, Sigma) at various cultural times up to 7 days. MTT assay is a rapid colorimetric method based on the mitochondrial succinate dehydronase conversion of tetrazolium to MTT formazan to determine quantitatively the number of viable cells. Briefly, the cell-containing scaffolds were rinsed with serum-free culture medium to remove the unattached cells and then transferred to another cell culture plate which contained 100μL 0.5 mg/mL RPMII 1640 solution. The cells on the scaffolds were incubated at 37°C for 4h for the MTT formazan formation. The MTT formazan were dissolved by 300μL dimethyl sulfoxide (DMSO), and then transferred to a 96-well plate. The optical density (OD) of the MTT formazan solution was measured with an automatic microplate reader (ELX 800, Bio-Tek) at a wavelength of 570nm referred to the adsorption at 630 nm. DMSO solution served as a blank and PCL film was assayed as well for the background control. Four parallel replicates were measured for each sample.

**Cell Morphologies**

The cell-containing scaffolds were replenished with fresh culture medium every second day. After being cultured for 1, 3, and 7 days, the scaffolds were washed with PBS three times. The cells on the scaffold were fixed with 5% glutaraldehyde in PBS for 24h at 4°C. The samples were then dehydrated sequentially in 50, 70, 90, and 100% ethanol for 15 min respectively, freeze-dried, sputter-coated with gold, and examined in a scanning electron microscope.

**Statistical Analysis**

Experiments were run at least in triplet in parallel tests. Data are expressed with standard deviations (SD) for \( n \geq 3 \). Statistical evaluation was performed by one-way analysis of variance for multiple
comparisons using the Bonferroni procedure. The mean values were considered to be significantly different when the probability of difference fell below 5% (i.e., $P < 0.05$) or 1% (i.e., $P < 0.01$).

RESULTS AND DISCUSSIONS

Scaffold Characterizations

Adsorbed CS Content and Stability on the Scaffold

Based on the calculation from Expression (2), CS contents between 5.7 and $9.2 \pm 0.1 \text{ wt\%}$ were achieved in the various CS-scaffolds with pore sizes ranging from 40 to $125 \text{ \mu m}$ (Table 1). Figure 1 shows the CS contents in the PCL scaffold modified with 1.0% CS solution. It is found that the adsorbed CS content does not depend considerably on the pore size of the scaffold, the porosity of the scaffold being determined by the amount of porogen used. When the amount of NaCl particles used were constant, CS adsorption content will remain at the same level. On the other hand, when the pore sizes of scaffold were fixed, for instance, at $75–125 \text{ \mu m}$, the mean porosity of scaffold were constant, the adsorbed CS contents would be changed by a small amount with different CS concentration solutions. It can be seen in Figure 2 that the adsorbed CS contents were increased a little from 6.6 to $8.4 \pm 0.1 \text{ wt\%}$ as the CS solution concentrations were increased.

![Figure 1](image-url)

**Figure 1.** The dependence of adsorbed CS content (wt\%) in the PCL scaffold (modified with chitosan concentration of 1.0% solution) on the pore size.
from 0.5 to 2.0% (g/mL), and reached a saturation level when the CS solution concentration exceeded 2.0%.

Figure 3 shows the changes in the ratio of CS-retention ($R_t$) in the porous CS-modified scaffold treated in physiological-like conditions up to 7 days. The $R_t$s for all the scaffolds were higher than 80% after 7 day’s immersion in PBS solution. The slight decrease in $R_t$s after 1 day’s soak maybe due to the detachment of some CS fragments from the scaffolds. The achieved high $R_t$ indicates that CS adsorption on the PCL scaffold remains stable under our experiment conditions.

Scaffold Morphologies

SEM observation in Figure 4 demonstrates the successful coating of CS on the PCL scaffolds fabricated by a NaCl porogen with 100–200 meshes. There is a thin CS layer on the surface of the pores in the porous scaffolds. The pore sizes of CS-modified PCL scaffold are somewhat reduced compared with the porous PCL scaffold (Figure 4(a)), but there are no significant differences in the CS-modified scaffolds treated with CS solutions with concentrations ranging from 0.5 to 2.0%, which may be explained by the results of the slightly increased CS-adsorbed weight, as discussed above.

**Figure 2.** The dependence of adsorbed CS content (wt%) in the PCL scaffold (with pore size of 75–125 μm) on the CS solution concentrations.
Figure 5 shows the SEM images of cross sections of 1.0% chitosan solution modified PCL scaffolds. There is an obvious change trend in the pore sizes coated with CS. The CS-coated pore sizes are reduced by the order of 100\( \times \)25, 75\( \times \)20, 50\( \times \)20, and 30\( \times \)10\( \mu \)m in Figure 5(a)–(d), respectively, as the original pore sizes of PCL scaffolds fabricated by NaCl porogens were decreased from 80 to 300 mesh. This shows that the larger the original pore size of the PCL scaffold, the bigger the pore size of CS-modified scaffold would be, after CS coating. The CS coating merely reduces the pore size of the original PCL scaffold by a small amount with the studied CS concentration.

On the other hand, although it is found that there is some graphic difference in Figure 5 for those PCL scaffolds fabricated via the same procedure and with a similar amount of adsorbed chitosan, it could be explained in the following manner. In fact, the obtained amount of the adsorbed chitosan on the PCL scaffold in Figure 2 is an average
value evaluated from five parallel experiments. However, the sample for SEM measurement in Figure 5 was only one of these five experiments, so the individual SEM micrograph in Figure 5 may not be quite identical to the others, even though, we could find a similar influence of chitosan on the surface morphology of the scaffold. In addition, because the used PCL scaffolds are porous, and the used chitosan solution are dilute, we thought the absorbed chitosan could enter deeply into the porous PCL scaffold body which is demonstrated by the cell growth in this work.

Figure 4. SEM images of surfaces of CS-modified PCL scaffolds treated with CS solutions at concentrations of 0 (a), 0.5 (b), 1.0 (c), 1.5 (d), and 2.0% (e), respectively. The inserted images were viewed at higher magnification. PCL scaffolds were fabricated with 100–200 mesh of NaCl porogen.
Cell Culture

Cell Proliferation on the Scaffolds

Cell adhesion and proliferation are dependent on the interaction between the cells and the biomaterial scaffold. The result of MTT assay for 1-day cell cultures shows that there are more cells adhered on the CS-modified PCL scaffold than the control (Figure 6(a)–(c)) except for the smaller pore size of 30–50 µm (Figure 6(d)). These results suggest that chitosan-modified PCL scaffolds are more favorable for cell attachment. The lower number of cells attached on the scaffold with the smaller pore size (Figure 6(d)) is possibly due to the fact that small chitosan-coated pores may prevent the cells from entering inside the scaffold. The scaffold with large pores will allow more cells into the scaffold body. In addition, it is noted that after 7-days culture, there is a remarkable increase in the cell numbers on the CS-modified PCL scaffolds compared with the control (Figure 6(a)–(c)). This means that the chitosan modification for the PCL scaffold also significantly improves the cell proliferation. Therefore, chitosan coating on the PCL scaffold enhances the cellular attachment and proliferation. In our work,

Figure 5. SEM images of modified PCL scaffolds with 1.0% CS solution. The porous PCL scaffolds were fabricated with NaCl porogens of 80–120 mesh (a), 120–150 mesh (b), 150–200 mesh (c), and 200–300 mesh (d), respectively.
the cell numbers adsorbed on the different scaffolds are calculated by MTT assay, which involves two processes. One is to extract the purple formazan from the porous scaffold, and the other is to dissolve the extracted formazan into DMSO solution for the optical density measurements. However, it is difficult to extract completely the formazan from the scaffolds, especially from those with smaller pores. Therefore, it is not easy to compare quantitatively the cell numbers among the scaffolds with different pore sizes in this work, even though we thought that the adsorbed cell amount would be dependent on the pore size of the scaffold as well as the scaffold modification shown in Figure 6.

**Cell Morphologies**

The cell morphologies on the PCL and CS-modified PCL scaffolds are shown in Figure 7. The cells look like spheres on both types of
Figure 7. SEM images of fibroblasts after different number of days’ culture on the scaffolds. Cells attached on the surface of PCL (a-1) and CS-modified scaffold CS/PCL (a-2) for 1 day; cells proliferated on the surfaces of the PCL scaffold (b-1) and CS/PCL scaffold (b-2) for 3 days; cells proliferated in the pores of PCL (c-1) and CS/PCL scaffolds (c-2) for 3 days; and cells cover the PCL (d-1) and CS/PCL (d-2) scaffolds after 7-days culture.
scaffolds at the first day (Figure 7(a-1) and (a-2)). After 3-days culture, the cells proliferate not only on the surface of the scaffold (Figure 7(b-1) and (b-2)), but also inside the pores (Figure 7(c-1) and (c-2)). The cells maintain a shuttle-like morphology. After culturing for a week, the cells almost cover all the surface of the scaffolds (Figure 7(d-1) and (d-2)). The cells on CS-modified scaffolds are much more elongated and stretched out (Figure 7(d-2)). It is clear that the cells adhere more firmly on the CS-modified scaffold. These results also indicate that CS modification dramatically enhances the cellular affinity and compatibility for the PCL scaffold.

CONCLUSIONS

In this work, we studied the morphologies and biocompatibility of the chitosan-modified PCL scaffolds. The scaffold morphology is affected by only a small amount by the chitosan solution with concentrations ranging from 0.5 to 2.0%. The adsorption content of CS on the scaffold depends only on the porosity of scaffold rather than the pore size of the scaffold. Importantly, the cell adhesion and proliferation on the PCL scaffolds are considerably improved after CS modification. The results suggest that CS modification efficiently enhances the biocompatibility of PCL materials. The present work with a simple but efficient way to modify PCL develops the potential application of PCL in the field of tissue engineering.

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