Preparation and Characterization of Poly(l-lactic acid)-Chitosan Hybrid Scaffolds With Drug Release Capability

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Abstract: Novel poly(l-lactic acid) (PLLA)–chitosan hybrid scaffolds were developed in order to be used as tissue-engineering scaffolds and drug release carriers. The incorporation of chitosan into the PLLA porous structure allows for producing chitosan-based scaffold devices with interesting damping and stiffness aimed at being used in tissue engineering of bone or cartilage. The pore structure of the hybrid scaffolds was influenced by the concentration of the chitosan solution introduced into the PLLA scaffold. For lower concentrations, chitosan was mainly deposited onto the PLLA surface, whereas for higher concentration chitosan formed also microfibrilar structures within the pore walls of the PLLA foam that may act as additional soft anchorage sites for cells. Equilibrium water uptakes up to about 110% were achieved in 24 h. An anti-inflammatory drug, ketoprofen, was loaded within the chitosan component of the hybrid scaffolds by immersing the scaffolds in a drug–ethanol solution. The drug was released sharply within the initial periods (~2–4 h), but the rate decreased further, showing a sustained release. The drug release rate can be controlled by the chitosan content and cross-link densities, suggesting the effectiveness of the hybrid scaffold as a drug delivery system. © 2006 Wiley Periodicals, Inc. J Biomed Mater Res Part B: Appl Biomater 81B: 427–434, 2007

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INTRODUCTION

Tissue engineering is a rapidly upcoming discipline with the intention to repair, replace or regenerate injured tissues and organs. In tissue engineering, the scaffolds play a key role as they serve as a three-dimensional template for cell differentiation, adhesion, proliferation, and formation of an extracellular matrix, as well as a carrier of the growth factors or other biomolecular signals.1 Together with the choice of appropriate cells and bioactive agents, the suitable material for the scaffold preparation plays a crucial role for the success of the application.2–4 In this context, considerable attention has been given to chitosan because of its low cost, large-scale availability, antimicrobial activity, low toxicity, biodegradability, and biocompatibility.5–10 Chitosan is a deacetylated derivative of chitin, commonly found in shells of marine crustaceans and cell walls of fungi. In tissue engineering applications, the cationic nature of chitosan is primarily responsible for electrostatic interactions with anionic glycosaminoglycans, proteoglycans, and other negatively charged molecules. This property is of great interest because a large number of cytokines/growth factors are linked to glycosaminoglycans, and a scaffold incorporating a chitosan–glycosaminoglycans complex may retain and concentrate growth factors secreted by colonizing cells. Moreover, the N-acetylglucosamine moiety in chitosan holds the specific interactions with growth factors, receptors, and adhesion proteins.11,12

Chitosan has been combined with a variety of delivery materials such as alginate, hydroxyapatite, hyaluronic acid, calcium phosphate, poly(methylmethacrylate), and growth factors for potential application in tissue engineering.13–18 However, the poor mechanical property of chitosan limits its usage in the field of tissue engineering of hard tissues.19
Poly(L-lactic acid) (PLLA) is a biodegradable and easily processable synthetic polymer and has good mechanical properties. Because of these characteristics, it has been proposed for tissue engineering applications. However, PLLA shows undesirable host reactions such as inflammatory and allergenic reactions because of the decrease of local pH as a consequence of the hydrolytic degradation of PLLA. It would be interesting, then, to combine chitosan with PLLA, especially in the surface and inside the pores, so chitosan can interact more directly with cells whereas PLLA provides both mechanical strength and stiffness to the biodegradable structure. Moreover, due to its cationic nature, chitosan could act as a buffer to minimize the drop of pH resulting from the PLLA degradation. It is found that this kind of complex materials evoke a minimal foreign body reaction. This study explored a novel kind of hybrid porous scaffolds by introducing a chitosan environment, in terms of surface coating or formation of a soft porous scaffold, into the pores of a previously prepared PLLA scaffold, to be used in tissue engineering applications. A soft/low density scaffold of chitosan inside a porous PLLA structure could be helpful for biological aptitude to stimulate cell proliferation and histarchitectural tissue organization. The PLLA–chitosan hybrid scaffold could be potentially useful for tissue regeneration because of its combined benefits, that is, the biodegradability and good mechanical properties of PLLA and the biocompatibility and shape availability of chitosan. Moreover, the cationic and hydrogel characteristics of the chitosan component could be beneficial for drug administration. In this work, the morphological features, dynamic mechanical properties, and the swelling behavior of such chitosan/PLLA scaffolds were investigated.

In recent years, there has been an increasing interest to produce scaffolds that can deliver drugs such as antibiotics, antitumors, and growth factors. The utility of a scaffold as a drug delivery vehicle can be employed to initiate cellular processes that lead to the creation of a functional tissue that integrates with the body. For example, the release of tissue-specific growth factors can induce differentiation of endogenous or transplanted progenitor cells into the appropriate cell type. In this work, a model drug, ketoprofen, was encapsulated within the chitosan component of the hybrid scaffolds. The in vitro drug release from the scaffolds was then examined. Since the main goal of this work was to produce chitosan-based scaffolds with adequate mechanical properties, the viscoelastic properties of the hybrid scaffolds were analyzed using dynamic mechanical analysis (DMA). This technique measures the deformation response of the material under a cyclic load excitation, as a function of frequency or temperature, being adequate to probe the solid-state rheological properties of polymeric systems. It has also shown that this technique may be useful to extract relevant information on the performance of biomaterials. Among other things, DMA is able to inform about the damping capability of the material which provides an indication on the ability of the implant to absorb mechanical energy resulting from the movements of the patient.

**MATERIALS AND METHODS**

**Materials**

Chitosan (M_w, 20,000; degree of deacetylation, 75–85%) and ketoprofen were purchased from Sigma Chemical Company. PLLA (M_n, 69,000) was obtained from Cargill Dow. All other chemicals were of analytical grade and used without further purification.

**Fabrication of PLLA Scaffold**

The particle leaching method has been used to produce polymeric foams for tissue engineering applications. PLLA and NaCl particles with weight ratio of 20:80 (PLLA/NaCl) were homogeneously mixed and placed in a disk mould with 7 mm of diameter. NaCl particles with sizes between 300 and 800 μm were used to adjust the finished scaffold pore size. The PLLA/NaCl mixture was heated inside the mould between hot plates at 180°C, i.e. slightly above the melting temperature of PLLA, and compressed at 150 MPa for 10 min using a hydraulic press to yield solid disks. The NaCl particles were subsequently removed from the scaffolds by leaching the scaffolds in distilled water with shaking for 3 days.

**Fabrication of PLLA-Chitosan Scaffold**

Chitosan solutions (0.6, 0.8, and 1 wt %) were prepared by dissolving different amounts of chitosan in 1% acetic acid at room temperature under magnetic stirring for 3 h. With these solutions, predetermined amounts of glutaraldehyde were added and stirred for 30 min in order to achieve 0.01, 0.05, and 0.1 M concentration of glutaraldehyde in the chitosan solution. The previously prepared PLLA scaffolds were dipped into the crosslinked chitosan solution and then stirred for 30 min in order to ensure the complete penetration of chitosan into the pores of the PLLA scaffolds. The PLLA scaffolds mixed with chitosan solution were kept at −80°C for 3 h and then freeze dried. The dried PLLA–Chitosan scaffolds were washed with 0.1M NaOH solution in order to neutralize the scaffolds and freeze dried again. The composition and designation of the PLLA–chitosan hybrid scaffolds are summarized in Table I. Pure chitosan scaffolds were also prepared from 1 wt % chitosan solution using freeze-drying method.

**Scanning Electron Microscopy**

Samples were coated with gold using a Hitachi IB-2 coater at 6 mA for 6 min, and their pore structures were observed under a scanning electron microscope (SEM, Leica Cambridge S360) at an accelerating voltage of 15 kV.
Dry scaffolds were accurately weighed (25 mL of phosphate buffer solution (pH 7.4) at 37°C. The water uptake behavior of the scaffolds was studied in three steps: (i) Temperature scans, (ii) Frequency scans, and (iii) Stress-amplitude scans. The experiments were conducted at 37°C and 1 Hz frequency, where the dynamic stress was varied between 2.8 Pa and 28 kPa at a rate of 1.4 kPa/min. The static stress was fixed at a value 1.2 times the dynamic stress. These experiments were performed to evaluate the range in which the materials present a linear viscoelastic behavior. The complex modulus ($E^*$) was obtained as the slope of the stress strain curve in the linear zone. Then, the storage modulus was calculated using $E' = E^* \cos \delta$. Two of such experiments were performed for each material.

Dynamic Mechanical Analysis

DMA was carried out using Perkin-Elmer DMA7 equipment using the parallel plate measurement system. The samples dimensions were 4 mm depth, 6 mm width being the thickness 10 mm. Three kinds of experiments were performed:

(i) Stress-amplitude scans. These experiments were carried out at 37°C and 1 Hz frequency, where the dynamic stress was varied between 2.8 Pa and 28 kPa at a rate of 1.4 kPa/min. The static stress was fixed at a value 1.2 times the dynamic stress. These experiments were performed to evaluate the range in which the materials present a linear viscoelastic behavior. The complex modulus ($E^*$) was obtained as the slope of the stress strain curve in the linear zone. Then, the storage modulus was calculated using $E' = E^* \cos \delta$. Two of such experiments were performed for each material.

(ii) Frequency scans. The experiments were conducted at 37°C with frequency ranging between 1 and 25 Hz. For the hybrid scaffolds, a static force of 33 kPa and a dynamic force of 28 kPa was employed, whereas for pure chitosan foams such values were 1.1 and 0.5 kPa, respectively. Such load values allowed to obtain accurate viscoelastic data, as the strain amplitude was sufficiently high, while maintaining the measurements within the linear viscoelastic regime.

(iii) Temperature scans. The temperature scans were performed in the temperature range between 10 and 80°C and a heating rate of 3°C/min. The frequency was 1 Hz and the static and dynamic stresses were the same as those used in the frequency scans. Two temperature scans were performed for each material.

Determination of Water Uptake

The water uptake behavior of the scaffolds was studied in 25 mL of phosphate buffer solution (pH 7.4) at 37°C. The dry scaffolds were accurately weighed (~0.1 g) and immersed in buffer solution. At predetermined time intervals (4, 24, 48, 72, and 96 h) the swollen scaffolds were weighed after they were wiped with soft paper tissue. The degree of water uptake for each sample at time $t$ was calculated by using the expression: $(W_f - W_0)/W_0 \times 100$, where $W_f$ and $W_0$ are the weights of the scaffolds at time $t$ and in the dry state, respectively.

Drug Loading and Release

The drug, ketoprofen, was loaded by immersing precisely weighed amount of scaffolds ($5 \times 0.1$ g) in 5 mL of drug–ethanol solution (25 mg/mL) in a small glass vial for 48 h at room temperature. Then, the mixture was filtered. The concentration of ketoprofen solution after filtering was determined by using a spectrophotometer at 267 nm. The relative amount of loaded ketoprofen by the scaffolds $(A)$ was calculated from

$$A = V(C_0 - C_1)/W$$

where $V$ is the volume of ketoprofen solution (mL), $C_0$ is the initial concentration of ketoprofen (mg/mL), $C_1$ is the concentration of ketoprofen solution after adsorption (mg/mL), and $W$ is the weight of the scaffolds (g).

Drug loaded scaffolds (~0.1 g) were suspended in 50 mL of phosphate buffered solution at pH 7.4 contained in a glass bottle. This dissolution medium was stirred at 100 rpm in a horizontal laboratory shaker and maintained at 37°C in a water bath. Samples (2 mL) were periodically removed for testing and the volume of each sample was replaced by the same volume of fresh medium. The loss of drug content by doing so at each time point was calculated from the raw data and added it to the cumulative to get the correct drug release profile. The amount of released ketoprofen was analyzed with a spectrophotometer at 267 nm. The drug release studies were performed in triplicate for each of the samples.

RESULTS AND DISCUSSION

Morphology

Figure 1 shows the typical SEM morphologies of the PLLA [Figure 1(A)] and the PLLA-chitosan hybrid porous scaffolds [Figure 1(B–F)]. The pure PLLA scaffold showed a well-developed porous structure, consisting of open pore channels and inter connected framework. The porosity and pore size were measured to be approximately 70% and 300–800 μm, respectively. When the hybrid scaffold was prepared from PLLA with the low concentration of chitosan (0.6% w/v) and glutaraldehyde (0.01M), the scaffold typically maintained the control PLLA framework structure [Figure 1(B)]. Therefore, we may infer that the chitosan fraction will be mainly deposited onto the pores’ surface of the PLLA scaffold, which is consistent with the higher roughness of the surface seen in Figure 1(B), with respect to Figure 1(A). Note that, in this case, the interconnectivity

<table>
<thead>
<tr>
<th>Scaffold Type</th>
<th>Initial Chitosan Concentration (% wt/v)</th>
<th>Glutaraldehyde Concentration (M)</th>
<th>Drug Loading (mg/0.1 g Scaffold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H1</td>
<td>0.6</td>
<td>0.01</td>
<td>13.61</td>
</tr>
<tr>
<td>H2</td>
<td>0.8</td>
<td>0.01</td>
<td>13.74</td>
</tr>
<tr>
<td>H3</td>
<td>1</td>
<td>0.01</td>
<td>14.12</td>
</tr>
<tr>
<td>H4</td>
<td>1</td>
<td>0.05</td>
<td>13.91</td>
</tr>
<tr>
<td>H5</td>
<td>1</td>
<td>0.1</td>
<td>13.83</td>
</tr>
</tbody>
</table>
of the initial scaffold is apparently not compromised by the presence of chitosan. With increasing chitosan concentration the volume of the PLLA pores are occupied with a highly porous chitosan network. Note that even at the highest chitosan concentration the pores in the PLLA structure will always be filled by a low density and highly hydrated chitosan matrix that facilitate the diffusion of nutrients for cells and release of wastes. Chitosan concentration has a significant effect on the pore structure of the hybrid scaffolds. For intermediate concentrations, chitosan microfibers are formed inside the pores [see, e.g., Figure 1(C)] that will also act as additional anchorage sites for cell attachment. Similar morphologies were found in PLLA–collagen, chitosan–gelatin/β-tricalcium phosphate and hydroxyapatite/chi-

Figure 1. SEM morphologies of the hybrid scaffolds (A) PLLA without chitosan, (B) H1, (C) H2, (D) H3, (E) H4, and (F) H5. Bar = 200 μm.
tosan–gelatin hybrid scaffolds. The effect of glutaraldehyde concentration on the cross sectional morphology of hybrid scaffolds is shown in Figure 1(D–F). The interconnected 3D porous structure of the scaffolds was retained even after the treatment with higher concentration of glutaraldehyde; however, while increasing the concentration of glutaraldehyde, chitosan formed a membrane-like morphology inside the pores of PLLA.

**Mechanical Properties**

From the DMA experiments with varying dynamic stress amplitude it was possible to conclude that all the scaffolds under study showed a linear viscoelastic behavior up to 33 kPa. Therefore, both frequency and temperature scans experiments were conducted in the linear regime. The storage modulus of the pure chitosan foam is about 0.1 MPa at 37°C, which makes it difficult to use in tissue engineering applications where superior mechanical properties are required. Moreover, it is expected that this values drops even more considerably in physiological environment as the polymer is highly hydrophilic. Figure 2 clearly shows that the incorporation of chitosan into the PLLA structure is an adequate way to obtain chitosan-based scaffolds with highly improved stiffness. In fact, in this case, storage moduli above 7 MPa were measured in the hybrid foams in the frequency range analyzed, at 37°C; note that the frequency interval (1–25 Hz) is meaningful if one considers the typical time-scales of the movements occurring in the body. Only results from the H2 scaffold are presented, but values of the same order were observed for the other formulations (see comparison later).

A direct comparison between the mechanical performance of pure chitosan and hybrid scaffolds, for the particular case of H4, can be seen in Figure 3, obtained from temperature scans, at a fixed frequency of 1 Hz. Both \( E' \) and \( \tan \delta \) are quite stable for the pure chitosan scaffold, being the loss factor higher than for the hybrid foam around 37°C. This indicates that the damping properties of chitosan are higher than in PLLA. In fact, complementary measurements performed in chitosan foams lead to values around \( \tan \delta = 0.14 \), i.e., about five times higher than the corresponding PLLA scaffold.

For the hybrid scaffold, the sudden decrease of \( E' \) in Figure 3, accompanied by the peak in \( \tan \delta \) above 50°C, is assigned to the glass transition of PLLA. The glass transition temperature of the hybrid foams, estimated here by the temperature of maximum \( \tan \delta \), was shown to be independent of the chitosan content and cross-linking: it varied between 56.6 and 57.7°C, without any particular trend with the materials’ features. Therefore, as expected, there is no interaction at the molecular level between the two materials in the hybrid scaffolds. To better understand the influence of chitosan on the viscoelastic properties of the foams, both \( E' \) and \( \tan \delta \) were compared for the different formulations, at 37°C and 1 Hz. Figure 4 shows these results, where both parameters were represented by averaging the values from the three kinds of experiments performed. Note that, for any given material, no particular systematic differences were detected either in \( E' \) nor in \( \tan \delta \) when the different experimental protocols were used.

Figure 4 shows that the addition of chitosan in PLLA did not modify noticeably the storage modulus; in fact the results obtained with the different formulations are not statistically significant. Again not being significant, the loss factor of the hybrid scaffolds seems to be slightly increased with respect to the PLLA foam. This result is related to the higher loss factor of chitosan, as noted earlier. Note that for H3, H4, and H5, a continuous decrease in \( \tan \delta \) is observed as increasing cross-linking enhances the elastic component of the viscoelastic response. The general increase of the loss factor with the introduction of chitosan, in comparison with pure PLLA scaffold, may be relevant in the use of such hybrid systems in biomedical applications: for orthopedic purposes the implanted materials should have preferably high value of \( \tan \delta \), because bone

![Figure 2](image1.png)

**Figure 2.** Dynamic mechanical behavior as a function of the frequency at 37°C for the scaffold H2. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

![Figure 3](image2.png)

**Figure 3.** Dynamic mechanical behavior of both H4 (solid lines) and chitosan foam (CH-dotted lines) at 1 Hz, obtained during temperature scans. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]
also exhibits a clear viscoelastic behavior, with tan δ ranging between 0.02 and 0.03 at meaningful frequencies. This will allow the material to dissipate to a larger extent the cyclic mechanical energy imposed and to integrate better with the surrounding tissue that also exhibit tissue-dependent mechanical behavior.

Water Uptake Behavior

The ability of a scaffold to preserve water is an important aspect to be investigated for tissue engineering and drug delivery applications. The water uptake percent of hybrid scaffolds with different concentration of chitosan and cross-link densities at pH 7.4 are given in Figure 5. As seen here, the scaffolds did absorb water rather fast in about 4 h, and then gradually reached equilibrium in about 24 h. The water-binding ability of the hybrid scaffold could be mainly attributed to the hydrophilic nature of chitosan present in the scaffold. In general, the water uptake decreases as the cross-linking degree is increased because of the decrease in the number of hydrophilic groups as well as the more difficulty in the structural expansion due to the more dense covalently linked network. For the specific case of chitosan, a more complex relationship is found between cross-linking degree with glutaraldehyde and water uptake capability, because the crystalline content in the material is also changing. The results in Figure 5 indicate that the percent of water uptake increases with increasing chitosan concentration or with decreasing cross-link density. The maximum water uptake (110%) was achieved by using the highest concentration of chitosan (1%) with the lowest cross-link densities. The water uptake of the scaffold prepared by using 0.6% chitosan and 0.01M glutaraldehyde was only about 45%. Figure 5 also shows the water uptake of control PLLA scaffold. Here we found only about 25% of water uptake, which was mainly due to the capillary action of pores present in the PLLA scaffold.

Drug Loading and Release

Table I shows the ketoprofen loading efficiency of the hybrid scaffolds at different conditions. The drug loading was increased by increasing the chitosan concentration or by decreasing the cross-linking densities. The highest drug loading (14.12 mg/0.1 g scaffold) was achieved by the scaffold that was prepared from 1% chitosan initial concentration and 0.01M glutaraldehyde concentration. It is obvious that the drug loading is increased by using the higher chitosan concentration in the scaffold, because it was possible to entrap more drug molecules on the large polymeric surface area by electrostatic interaction. Moreover, the higher water uptake capability of the scaffold which was cross-linked with the lower concentration of glutaraldehyde solution (0.01M), also facilitates the drug loading efficiency.

The drug release rate from hybrid scaffolds could be controlled by varying both chitosan and glutaraldehyde concentration. Figure 6 shows the drug release profile of the hybrid scaffolds prepared at different conditions. The results showed that the drug release rate was quite fast at the initial period of time. About 45–70% of the drug was released in about 2 h. Such an abrupt release was due to the free drug remaining at the surface without entrapped efficiently within the polymer matrix. After this initial burst the drug was released in a sustained manner, and the rate decreased for all the hybrid scaffolds with time. Such a release profile observed in the hybrid scaffolds could provide a rapid delivery of drug to give anti-inflammatory effects at the wound site and a further sustained release to aid long-term healing. In this study, it is observed that the water uptake curves are quite similar to the release curves. It seems that release obeys a swelling-controlled mechanism, especially at the initial period of release. After the initial period, in which the equilibrium water uptake is achieved, the release is most probably followed by a diffu-
sion-controlled mechanism. This is an expected behavior found in the case of chitosan scaffold during drug release studies; first a hydration followed by polymer chain relaxation takes place by water penetration into the matrix. During this process, high release rates occur because of the presence of the drug at and near the surface. After forming the gel structure the release continues with the diffusion mechanism through the matrix by a much slower release rate. This typical phenomenon is the same in the case of drug release from PLLA–chitosan scaffolds. Other chitosan derivatives could be used to deliver bioactive agents at a slower rate. For example, it was found that the delivery of ketoprofen could be retarded in chitosan-based particulate systems, bearing β-cyclodextrin groups. In the present study, it was found that the profile of drug release was faster at lower pH for all formulations (data not shown). This is an expected behavior, and reported elsewhere for chitosan particles loaded with the same drug, as chitosan becomes soluble at pH lower than ~6 as a result of the amine protonation. Therefore, the developed scaffolds may also have a pH responsive capability.

The amount of drug release was found to increase with the decrease in the concentration of chitosan in the hybrid scaffold. At the lower concentration of chitosan, the less ionic interactions between chitosan and drug molecules might explain this observation. The highest drug released fraction was observed in the case of the scaffold prepared by using the lowest amount of cross-linking agent. It seems that crosslinking density has an influence on the fraction of the drug initially loaded that can be released. These results showed that PLLA–chitosan scaffold can be suitable for sustained drug release in the tissue engineering approaches. It is also interesting to note that one more kind of drug could be incorporated within the PLLA matrix of the hybrid scaffold. This would allow for the production of constructs that could release two different bioactive substances (drugs, enzymes, proteins) with two different time scales. The combination of PLLA–chitosan hybrid scaffolds with sustained drug (e.g., protein, DNA) delivery may be used to enhance both the inductive and cell transplantation approaches to tissue engineering, which are based on the ability to direct cellular processes within a developing or regenerating tissue.

CONCLUSIONS

The PLLA–chitosan hybrid scaffolds were prepared by using PLLA with different concentrations of chitosan and glutaraldehyde. The concept consisted here is to create a chitosan porous structure, in which the cells and tissues would mostly interact, within the pore structure of a stiffer PLLA scaffold. The concentration of chitosan and glutaraldehyde had an influence on the morphology, mechanical, and swelling property of the hybrid scaffolds. The hybrid foams exhibit a much higher stiffness than pure chitosan foams, indicating that this strategy may allow for the use of chitosan-based structures in tissue engineering applications requiring some mechanical features. Moreover, the hybrid constructs presented significant damping characteristics, which may be beneficial in orthopedic applications. The percent water uptake of the hybrid scaffolds increased with either increasing chitosan content or decreasing crosslink densities. The maximum water uptake of the scaffolds (around 110%) was achieved in about 24 h. The developed scaffolds were found to be adequate to release previously loaded drugs. The drug release profile was affected by the composition of the scaffolds. The drug release was fast at the initial period, in agreement to the water uptake behavior; however, almost constant drug release was observed at the later time period. The hybrid scaffolds prepared with the lowest amount of chitosan and cross-linking agent showed the highest percent of drug release. These preliminary results suggested that PLLA–chitosan hybrid scaffolds could be suitable to release bioactive components for stimulating cell differentiation and proliferation or drugs, such as anti-inflammatories and antibiotics, to induce therapeutic effects in tissue engineering strategies.
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