Hydrogel-Filled Polylactide Porous Scaffolds for Cartilage Tissue Engineering

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Abstract: Polymer porous scaffolds and hydrogels have been separately employed as analogues of the native extra-cellular matrix (ECM). However, both of these two kinds of materials have their own advantages and shortcomings. In this work, an attempt to combine the advantages of these two kinds of materials is carried out. Poly-L-lactide (PLLA) scaffolds with good mechanical properties were prepared by thermally induced phase separation, which were then filled with hydrogel aiming at entrapment of cells within a support of predefined shape. Agar, which has a function to promote chondrogenesis, was selected to entrap chondrocytes, acting as analogues of native ECM. A straight forward merit of this construct is that both mechanical strength and macroscopic shape, and analogous ECM can be simultaneously achieved. The morphology and distribution of the chondrocytes were studied by confocal laser scanning microscopy (CLSM) and scanning electron microscopy (SEM). The cell growth behaviors were determined by MTT assay and collagen and glycosaminoglycan (GAG) secretion. After culture for 7 and 14 days, the cells in the construct were round and surrounded by the hydrogel. The MTT viability and the cell secretion in the chondrocytes/agar/scaffold construct were also higher than that of the chondrocytes/scaffold construct (control). Gelatin was further introduced into the construct, yielding improved GAG secretion and cytoviability. After implantation in the subcutaneous dorsum of nude mice for 4 weeks, cartilage-like specimens maintaining their original rectangular shapes were harvested. Histological examination showed that new cartilage was regenerated and a large quantity of collagen and GAG were secreted, while the cells in the control PLLA scaffold turned to be fibroblast-like with less secretion of extracellular matrices. The method provides a useful pathway of scaffold preparation and cell transplantation, which can achieve suitable mechanical properties and good cell performance simultaneously.

Keywords: cartilage tissue engineering; polylactic acid; scaffold; hydrogel; agar

INTRODUCTION

Cartilage degeneration, due to arthritis or sport injury, is a serious problem that affects health of people of all ages. Because cartilage shows very little tendency of self-repair, the injuries are maintained years and can eventually lead to further degeneration.1–3 An American study reported that arthritis accounted for 744,000 hospitalizations and 44 million ambulatory care visits in the United States in 1997.4 In China more than 10% people suffer from joint pain.

Nowadays tissue engineering has appeared as a new approach in treatment of damaged or lost cartilage via delivering isolated chondrocytes to the injured site to promote tissue integration.1 Artificial extra-cellular matrix (ECM) and the delivery of isolated chondrocytes to the matrices, which affect the success of the therapy, have been studied carefully in many previous works.2,5–10 Two kinds of materials have been generally utilized as the matrices so far: porous scaffolds and hydrogels. Accordingly the methods of cell transplantation are established too.

Polymers such as poly (glycol acid) (PGA), polylactide (PLA), and their copolymers (PLGA) and PCL and so forth are the primary scaffolding materials in various tissue engineering applications, including bone and other mineralized
tissues. These materials can easily form solid, stable porous structures to serve as predesigned three-dimensional scaffolds via various methods such as particle leaching, phase separation or 3D printing. They generally have good mechanical properties and their degradation rate can be mediated by their composition or molecular weight, which is one of the basic requirements of tissue engineering. However, in many previous researches, it has been shown that at first most cells tend to adhere and spread only on the surfaces of the pores with one side toward culture medium and the other side toward polymers, then form a monolayer cell sheet [Figure 1(a)]. Therefore, the morphology, amount, and distribution of cells are different from those of the native cartilage. For instance, Miller and Benya et al found that after growth in the state of monolayer for more than 1 month, the shape of chondrocytes changes into flat, amebocyte-like or fibroblast-like, and the secretion of type I collagen increases instead of type II collagen.

Chondrocytes in native tissue are normally rounded, and surrounded by matrix comprising collagens, glycoproteins, proteoglycans and hyaluronan. Therefore, hydrogel scaffolds are highly recommended in cartilage regeneration because they are highly hydrated three-dimensional networks that provide a nice place for cells to adhere, proliferate, and particularly differentiate. They can also provide chemical signals to the cells through incorporation of growth factors, and mechanical signals by manipulating the mechanical properties of the materials. On the other hand, the structure of hydrogels can mimic the *in vivo* cell growth environments of cartilage, which is a highly hydrated matrix comprising mainly of type II collagen and GAG. Agar is often used in biochemistry and bioengineering. It is mainly composed of agarose and agarpectin. Agarose gels seeded with cells have been used to investigate the efficacy of cellular-agarose composites in articular cartilage repairing in animal models. Previous works have shown that even after chondrocytes propagated as a monolayer of dedifferentiation as a consequence of passage, when propagated as a suspension culture in agarose gels they re-expressed a differentiated phenotype as assessed by re-expression of type II collagen and synthesis of proteoglycans with a level comparable to primary chondrocytes. However, poor mechanical strength of hydrogels cannot afford heavy load and is less convenient for surgical manipulation, thus limiting their applications in practice.

Assembly of these two kinds of scaffolds into a system would be a wise way to maintain their each advantages, while avoids shortcomings. One can expect that the cells/hydrogel/scaffold construct will have the following characteristics [Figure 1(b)]: (1) mimicking the physical growth environment of the native tissues such as cartilage, (2) avoiding surface modification of those cell-incompatible materials.
scaffold, (3) suitable mechanical strength for discretionary cutting, storage, and surgical operation conveniently, (4) incorporating water-soluble biomacromolecules, growth factors, and/or plasmid DNAs easily without losing their bioactivity, and thus (5) suspending the chondrocytes throughout the pores instead of adhering on the pore surfaces to endow the cells with round shape and normal phenotype. Exemplified here with poly-L-lactide (PLLA, the so called “hard scaffold”) and agar (the so called “soft scaffold”), we introduce here the effect of this combined system on behaviors of chondrocytes cultured in vitro and cartilage formation cultured in vivo.

MATERIALS AND METHODS

Materials

The PLLA (\(M_n = 200,000, M_w = 400,000\)) was synthesized using the method described previously.\(^{22}\) Agar (Bio Basic) and gelatin (Shanghai Chemical Industries, China) were dissolved in triple-distilled water or phosphate buffered saline (PBS) and sterilized in autoclave at 120°C for 15 min.

Scaffold Preparation and Microstructure Observation

PLLA scaffolds were manufactured using thermally induced phase separation (TIPS) method described previously.\(^{23–25}\) Scaffolds with pore diameters above 150 \(\mu m\) were prepared through the following steps. The 5% PLLA/1,4-dioxane-H\(_2\)O (87:13v) solution in a glass mold (1.2 cm in diameter and 15 cm in height) at 70°C was quickly quenched to 25°C, followed by coarsening at this temperature for 5 h to form sufficient phase domains. To obtain the scaffolds with a pore diameter of \(~350 \mu m\), two-step coarsening was adopted.\(^{23}\) In brief, after coarsened at 25°C for 5 h, the system was cooled down to 20°C and coarsened again for another 2.5 h. The solvents were then solidified at −20°C for 2 h, and were removed by freeze-drying.

In order to observe their inner microstructure under scanning electron microscopy (SEM, JEOL JEM-200CX, Japan), the porous scaffolds were fractured in liquid nitrogen. The diameter of the pores was determined and averaged from the SEM images by graph calculation software, SMile view. Diameters of the pores were determined and averaged from the SEM images by graph calculation software, SMile view.

Hydrogel Introduction

To load the hydrophilic biomacromolecule solution, the hydrophobic scaffolds were damped firstly with 75% alcohol solution for 1 day, followed by exchanging the alcohol with PBS (pH 7.4) for 12 h. The agar solution and cell suspension were mixed and dropped onto the surface of the wet scaffold. By the effects of gravity force and capillary force, the mixture was spontaneously absorbed into the scaffold. To induce the gelation, the constructs were quenched from 40 to 25°C for 15 min.

To observe the distribution of the gels in the PLLA scaffolds under confocal laser scanning microscopy (CLSM, Bio-Rad Radiance 2100), agar solution was initially mixed with 0.05% fluorescein sodium solution. The efficiency (\(R\)) of hydrogel introduction was calculated by the following equation:

\[
R = 100\% \times \frac{W_m - W_0}{W_c - W_0}
\]

where \(W_0\) is the weight of the PLLA scaffold, and \(W_m\) and \(W_c\) are the measured and calculated weights of the scaffolds after hydrogel loading. The calculated weight is derived from the scaffold porosity and hydrogel concentration.

Mechanical Strength

Samples with cylindrical shape, that is ~11 mm in diameter and ~25 mm in height, either filled or unfilled with hydrogels, were compressed by a mechanical tester (ZWICK ROELL, Germany). The cross-head speed was set as 1 mm/min. The compressive modulus was determined from the compressive curve at the initial strain of 2–6%.

Cell Culture In Vitro

Chondrocytes were isolated from rabbit ears (New Zealand Rabbit) by digesting the cartilage chips with 0.2% collagenase II (Sigma) and cultured in F-12 HAM’s supplemented with 20% fetal calf serum (FBS), 300 mg/L glutamine, 50 mg/L vitamin C, 100 U/mL penicillin, and 100 U/mL streptomycin. The cell suspension was then seeded in 11 cm tissue culture dishes (Falcon, seeding density 2 × 10^4 cells/cm²) and incubated in a humidified atmosphere of 95% air, 5% CO\(_2\) at 37°C.

After a confluent cell layer was formed (3–4 days), the cells were detached using 0.25% trypsin in PBS and were re-suspended in the supplemented culture medium as described above, and used for the experiments. Before cell seeding, the scaffold with an average pore diameter of 350 \(\mu m\) was sterilized using 75 vol% ethanol for 1 h and then incubated in PBS for 1 day to exchange the ethanol. Chondrocyte suspension (1 mL) (5 × 10^6 cells/mL) was mixed with 1% agar and the mixture was dropped directly onto the surface of the scaffold using a syringe. In order to assess chondrocyte growth behaviors, 0.01 g/mL gelatin was optionally added into the cell suspension too. After gelation, the cell-containing scaffold was then incubated in the culture medium under the conditions as described above. As a control, PLLA scaffold without agar was also seeded with the same density of chondrocytes and cultured similarly.
Cell Distribution and Morphology Observation

To observe the cell distribution under CLSM, the cell-containing scaffold was taken out from the culture plate and rinsed with PBS gently. Then 5 μg/mL fluorescein diacetate (FDA)/PBS solution was slowly injected into the scaffold. After incubated for 10 min the scaffold was gently cut into several parts. By this fluorescently staining, only the viable cells in the scaffold can be visualized under CLSM.27 To observe the morphology of the chondrocytes, the samples were fixed with 2.5% glutaraldehyde solution at 4°C for 1 h, then were sequentially dehydrated in 75 and 95% aqueous ethanol solution, each for 15 min. They were further dehydrated for two times in absolute ethanol, for one time in acetone and for one time in isoamylacetate, each for 15 min. The samples were put in a pressure chamber with liquid CO2, and the CO2 was vaporized at a temperature above its critical point. The dried samples were coated with Au by a sputter coater and examined by SEM (Stereoscan 260, Cambridge).

Cell Viability by MTT Assay

Cell viability assay was performed after cultured for 3–28 days. The chondrocytes containing scaffolds were cut into small pieces and MTT (3-(4,5-dimethyl) thiazol-2-yl-2,5-dimethyl tetrazolium bromide, 5 mg/mL) solution was added into the scaffolds. After continuously cultured for 4 h, dimethyl sulfoxide (DMSO) was added to dissolve the formazan pigment reduced by viable cells. The mixture was centrifuged at 10,000 rpm for 10 min to ensure the complete separation of the formazan pigment from the scaffolds. The absorbance of the samples was determined by referring to a calibration curve of standard DNA. The total collagen was indirectly determined from hydroxyproline content after hydrolysis (6M HCl at 115°C for 18 h) and reaction with p-dimethylaminobenzaldehyde and chloramine-T using a spectrophotometer (UV-2450, Shimadzu) at 550 nm.31,32,29 To measure the sulphated glycosaminoglycan (GAG) content, Alcian blue dye was employed according to ref.33 The sample was cut into small pieces and dispersed in 2 mL 1% papain solution (w/v, with 0.09% EDTA and 0.04% cysteine as solvents) before maintained at 37°C for 24 h. Two milliliter Alcian blue 8GX (Sigma-Aldrich, ~1.4 mg/mL, dissolved in 0.1 mol/L sodium acetate) was then added into the mixture. After 10 min, the absorbance at 480 nm was measured by UV–vis spectrophotometer. The content of GAG secreted by the chondrocytes in the scaffolds was determined by referring to a calibration curve of chondroitin sulfate. Data were presented as mass of component per scaffold volume (in vitro) or per wet weight (in vivo) of sample.

Biochemical Analysis and Histological Evaluation

The surface water of the samples was adsorbed by a filter paper and the wet weight (Ww) was measured immediately. These samples were subsequently freeze-dried until a constant weight (Wf) was reached. These freeze-dried samples were then subjected to characterizations of cell, collagen and GAG content. The water content were defined as (Wf/Ww) × 100%. Each data were averaged from 3 parallel measurements, and expressed as mean ± standard deviation. The cell content in the samples was assessed by quantifying DNA (7.7 pg DNA/cell) in the constructs using the Hoechst 33258 dye (Sigma assay).28,29 Hoechst 33258 is a bis-benzimide derivative that binds to the AT-rich regions of double stranded DNA and is excited in near UV (350 nm) and emits in the blue region (450 nm). Samples were frozen at −20°C for 2 h, lyophilized for 24 h, and digested with 1 mL papain (1% w/v, with 0.09% EDTA (disodium ethylenediaminetetraacetic acid) as solvent) per sample.30 Shortly after 100 μL papain digest solution was added into 2 mL Hoechst 33258 solution (1 μg/mL), the fluorescence intensity at 450 nm was measured by a fluorescent spectrophotometer (RF-5301PC, Shimadzu). The content of DNA in the samples was determined by referring to a calibration curve of standard DNA. The total collagen was indirectly determined from hydroxyproline content after hydrolysis (6M HCl at 115°C for 18 h) and reaction with p-dimethylaminobenzaldehyde and chloramine-T using a spectrophotometer (UV-2450, Shimadzu) at 550 nm.31,32,29 To measure the sulphated glycosaminoglycan (GAG) content, Alcian blue dye was employed according to ref.33 The sample was cut into small pieces and dispersed in 2 mL 1% papain solution (w/v, with 0.09% EDTA and 0.04% cysteine as solvents) before maintained at 37°C for 24 h. Two milliliter Alcian blue 8GX (Sigma-Aldrich, ~1.4 mg/mL, dissolved in 0.1 mol/L sodium acetate) was then added into the mixture. After 10 min, the absorbance at 480 nm was measured by UV–vis spectrophotometer. The content of GAG secreted by the chondrocytes in the scaffolds was determined by referring to a calibration curve of chondroitin sulfate. Data were presented as mass of component per scaffold volume (in vitro) or per wet weight (in vivo) of sample.

Implantation In Vivo

Before implantation, the scaffolds were cut into rectangle shape (about 8 × 3 × 2 mm3) and seeded with chondrocytes, either filled with agar or not, were implanted subcutaneously in the dorsum of athymic nude mice (n = 3). The athymic nude mice were purchased from Shanghai China for animal experiments. Athymic male mice were obtained at 5 weeks and acclimated for 1 week before use. The cell-material constructs were harvested after in vivo cultivation for 4 weeks. Samples for histological evaluation were fixed in neutral buffered formalin, embedded in paraffin, and sectioned in cross-sections through the center of the implants (5-μm thick sections). The cross sections were routinely stained with hematoxylin and eosin, Masson’s Trichrome stain (green stain for collagen)34 and Alcian blue (blue stain for GAG).35 The sections were also used for the immunostaining of collagen type II by collagen type II antibody (PV-9000, Poly-HRP anti-Mouse/Rabbit IgG Detection system, Beijing ZHONGSHAN Biotechnology). The samples were immersed in PBS containing 3% H2O2 at 25°C for 10 min to block nonspecific reactions. Subsequently, the sections were incubated in anti-collagen type II antibody working dilution at 4°C overnight. After washed with PBS three times, the samples were incubated in anti-rabbit IgG antibody working dilution at 37°C for 20–30 min. Finally, the samples were incubated in 3,3’-diaminobenzidine tetrahydrochloride (DAB) solution (0.5 mg/mL, with 0.01% H2O2 and Tris-HCl buffer solution as solvents, pH 7.6) after the anti-rabbit IgG antibody working dilution was removed by PBS. Collagen type II was stained brown.

Statistical Analysis

Data from all studies were analyzed using the ANOVA. The significant level was set as p < 0.05. Results are reported as mean ± standard deviation.

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RESULTS

Scaffold Properties and Agar Loading Efficiency

SEM and CLSM observation, as shown in Figure 2, revealed the morphology of the scaffold prepared via TIPS method and the agar introduction into the scaffold. By controlling the coarsening time after phase separation, PLLA scaffolds with round shape pores and an average pore diameter of $\sim 150 \ \mu m$ [Figure 2(a)] or $\sim 350 \ \mu m$ [Figure 2(b)] were successfully fabricated by normal coarsening and 2 step-coarsening, respectively. Their porosities were both 93%. The loading of hydrogels was firstly evidenced by direct observation under CLSM, utilizing fluorescein-containing agar hydrogel as an example [Figure 2(c,d)]. The images show that the hydrogels (bright regions) were uniformly distributed through the entire scaffolds regardless of the pore diameters. This is quite understandable since the pores in both scaffolds are highly interconnected, which is important not only for hydrogel introduction, but also for cell infiltration and nutrient transportation. Yet their exact loading efficiency for hydrogels was different, with a higher value for the scaffold of larger pore size (Table I).

Mechanical Strength of the Scaffolds

Figure 3 shows the stress-strain curves of the PLLA scaffolds at either wet or dry state, or loaded with agar. At a strain below $\sim 10\%$, the stress increased almost linearly. When the strains exceeded this value, platform regions appeared for all the scaffolds. In the linearly increased region, the deformation extent is small and might be considered as elastic. Hence the compressive modulus could be calculated. A higher compressive modulus (3.38 MPa) for the scaffold at wet state was found than that at dry state (2.05 MPa). After loading with agar, the modulus was increased further to 5.16 MPa.

Figure 2. SEM images of PLLA scaffolds with pore diameters of (a) $\sim 150 \ \mu m$, and (b) $\sim 350 \ \mu m$. (c) and (d) are CLSM images corresponding to (a) and (b) after loading of agar, respectively. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]
In Vitro

When seeding cells into scaffolds, an inevitable problem is that the cell suspension flows away along with the medium. The hydrogel could avoid the cells escaping and thus numerous cells could be entrapped in the cells/agar/scaffold construct. To investigate whether cell-seeding efficiency into 3D porous scaffold is enhanced, the chondrocyte number in the cells/agar/scaffold construct was compared with that of the cells/scaffold construct (Figure 4). The chondrocyte number in the cells/agar/scaffold construct was significantly higher ($p < 0.05$).

CLSM observed lots of viable chondrocytes in both the control [Figure 5(a,b)] and the cells/agar/scaffold construct [Figure 5(c,d)] after in vitro cultured for 7 and 14 days. However, there were a relatively larger number of chondrocytes in the scaffold filled with agar. The chondrocytes distributed more homogeneously in this agar-filled scaffold too. The cell numbers increased along with the prolongation of culture time. The difference of cell morphology in these two kinds of scaffolds was further observed by SEM. The cells seeded in the control PLLA scaffold adhered, spread and nearly formed monolayers on the pore walls after 7 day culture [Figure 6(a,b)]. In the cells/agar/scaffold construct, however, most of the chondrocytes were entrapped in the agar gel and remained round shape [Figure 6(c,d)].

To demonstrate the feasibility of the current method in loading biomacromolecules or cell growth factors, gelatin was added into the agar/PLLA system which then formed a composite hydrogels. Figure 5(e,f) shows that more chondrocytes were found after in vitro cultured for 7 and 14 days compared with the control PLLA scaffold and the cells/agar/scaffold construct. SEM images [Figure 6(e,f)] show that the chondrocytes entrapped in the hydrogel of agar and gelatin had similar round shape with the cells in agar. Cell cilium, a typical feature of chondrocytes, were clearly visualized on some cell surfaces [Figure 6(e)].

Quantitative analyses of the major constituents within these three kinds of scaffold are summarized in Table II, in which the hydroxyproline represents the secreted collagen level. Except for the water content, the cell viability, hydroxyproline amount and GAG secretion within each kind of the scaffolds were significantly increased when the culture time was prolonged from 7 to 14 days. At the same culture time, for example 7 or 14 days, the level of cell viability, hydroxyproline amount and GAG secretion was increased according to the sequence of the control, the agar filled and the agar and gelatin filled scaffolds. Statistical analysis confirmed significant difference ($p < 0.05$) between each samples.

To further demonstrate the efficacy of the hydrogel-filling scaffold, the chondrocytes were cultured as a function of time, taking agar and gelatin filled PLLA scaffold as a typical example. Figure 7(a,b) show that both the chondrocyte proliferation and viability increased along with the culture time. Compared with the control sample, however, these val-

### Table I. Loading Ratio of Agar in the PLLA Scaffold

<table>
<thead>
<tr>
<th>Hydrogel Concentration (%)</th>
<th>Scaffold With Pore Diameter of 150 μm (%)</th>
<th>Scaffold With Pore Diameter of 350 μm (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>103.4 ± 8.6a</td>
<td>104.4 ± 5.7</td>
</tr>
<tr>
<td>2</td>
<td>74.4 ± 9.4</td>
<td>83.2 ± 6.9</td>
</tr>
</tbody>
</table>

Differences between hydrogel concentration for the same scaffolds are significant ($p < 0.05$). Difference between scaffolds of different pore diameters at the same hydrogel concentration are not significant ($p > 0.05$).

a Values above 100% was caused by the adhesion of agar on the scaffold surface.

### Stress (MPa)

![Stress-strain curves of the PLLA scaffolds with a pore diameter of 350 μm at different hydrated states, or agar loading.](a)

### Modulus (MPa)

![Compressive modulus derived from (a). Differences between different samples are significant ($p < 0.05$).](b)
ues in the agar and gelatin filled scaffold were always larger than that of the control at each time point ($p < 0.05$). To quantitatively compare the viability per cell, the MTT Abs was normalized by the cell numbers at each time point and is presented in Figure 7(c). It shows that the viability per $10^4$ cells in the cells/agar/gelatin/scaffold construct at all the time points is always larger than that of the control, indicating that the chondrocytes within the hydrogel-filled scaffold possessed stronger metabolism activity too.

**Implantation and Histological Examination**

The cells/agar/scaffold constructs were implanted subcutaneously in the dorsum of athymic nude mice, as shown in Figure 8(a). The PLLA scaffolds seeded with chondrocytes were also implanted as the control samples. The implants were harvested after 4 weeks. They were surrounded by thin vascularized capsules and were easily dissected from the subcutaneous tissue. No evidence of superficial infection or fistula formation was demonstrated in the experimental mice. The control and the experimental constructs remained their original rectangular shapes throughout the implantation period [Figure 8(b)].

The specimens were stained by hematoxylin and eosin. Chondrocytes in their natural round morphology were found in the experimental construct [Figure 9(a)]. The matrix sur-

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**Figure 4.** Cell number in the control and the agar filled scaffolds after *in vitro* culture for 1 day. Cell seeding density $5 \times 10^6$/mL.

**Figure 5.** CLSM images to show the chondrocyte distribution in the (a,b) control PLLA scaffold, (c,d) agar filled PLLA scaffold and (e,f) agar and gelatin filled PLLA scaffold. (a,c,e) *in vitro* culture for 7 days, and (b,d,f) for 14 days. Cell seeding density $5 \times 10^6$/mL. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]
Figure 6. SEM images to show the chondrocytes in the (a) control PLLA scaffold, (c) agar filled PLLA scaffold and (e) agar and gelatin filled PLLA scaffold after in vitro culture for 7 days. (b), (d), (f) are higher magnification of (a), (c), (e), respectively. Cell seeding density 5 × 10^6/mL.
rounding the chondrocytes was stained to purple color because of the basophilic mucus and proteoglycan, which is similar to the condition of native hyaline cartilage. On the other hand, the cells in the control sample were relatively small and the morphology was fibroblast-like [Figure 9(b)]. Histological staining for cartilaginous extracellular matrix of collagen by Masson’s Trichrome stain revealed that a mass of collagen surrounded the chondrocytes [Figure 9(c)]. Collagen type II was detected in the matrix by immunohistochemical staining [Figure 9(d)]. By intensity of Alcian Blue staining, GAG was found surrounding the chondrocytes [Figure 9(e)]. Table III summarizes the collagen and GAG contents in the experimental construct, native cartilage and the control sample. The collagen and GAG secretion by the

TABLE II. Major Constituents Within the PLLA Scaffold (Pore Diameter 350 μm)/Hydrogel/Chondrocyte Constructs Cultured In Vitro for 7 and 14 days

<table>
<thead>
<tr>
<th></th>
<th>Cells/Scaffold Construct</th>
<th></th>
<th>Cells/Agar/Scaffold Construct</th>
<th></th>
<th>Cells/Agar/Gelatin/Scaffold Construct</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>7 days</td>
<td>14 days</td>
<td>7 days</td>
<td>14 days</td>
<td>7 days</td>
<td>14 days</td>
</tr>
<tr>
<td>Water (wt %)</td>
<td>86.7 ± 0.6</td>
<td>87.1 ± 0.8</td>
<td>88.1 ± 1.2</td>
<td>87.5 ± 0.8</td>
<td>87.1 ± 1.7</td>
<td>86.8 ± 0.7</td>
</tr>
<tr>
<td>MTT Abs (cm⁻³)</td>
<td>6.5 ± 1.2</td>
<td>9.8 ± 2.3</td>
<td>9.55 ± 0.5</td>
<td>12.7 ± 1.5</td>
<td>14.4 ± 1.4</td>
<td>22.1 ± 4.1</td>
</tr>
<tr>
<td>Hydroxyproline (μg/cm³)</td>
<td>202 ± 19</td>
<td>292 ± 28</td>
<td>338 ± 41</td>
<td>374 ± 26</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>GAG (mg/cm³)</td>
<td>1.28 ± 0.1</td>
<td>1.78 ± 0.1</td>
<td>2.69 ± 0.47</td>
<td>3.02 ± 0.21</td>
<td>3.61 ± 0.48</td>
<td>3.87 ± 0.22</td>
</tr>
</tbody>
</table>

Differences between different constructs at same culture time are significant ($p < 0.05$) except for the water content where $p > 0.05$.

* Because the gelatin has a large amount of hydroxyproline, the secreted collagen could not be determined by this method.

![Figure 7](image-url)  
**Figure 7.** (a) cell number, (b) MTT assay, (c) viability per $10^4$ cells of the scaffold filled with agar/gelatin and the control scaffold as a function of culture time in vitro. Cell seeding density $5 \times 10^6$/mL. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]
chondrocytes in the experimental construct were significantly higher than that of the control ($p < 0.05$), which were already close to the values of native cartilage after a 4 weeks-culture.

**DISCUSSION**

In present study, we demonstrated that assembly of polymer porous scaffold and hydrogel into a system in cartilage regeneration can maintain their each advantages, while avoids shortcomings. A cell/scaffold construct was made by introduction of chondrocytes containing agar into the “hard” PLLA scaffold, which was then cultured in vitro.

The PLLA scaffold with good mechanical properties was prepared via TIPS method. Thermally induced phase separation is a spontaneous process to form polymer-rich phase and polymer-lean phase, in which subsequent solvent removal will produce polymer sponge with well interconnected and opened pore structure. The pore size can be controlled by adjusting the coarsening time and quenching method. It is worth mentioning that smaller pores with a diameter ranging from several μm to ~20 μm can also be clearly visualized on the pore walls [Figure 2(a,b)]. Such features ensure the infiltration of hydrogels and/or cells and the CLSM observation [Figure 2(c,d)] and hydrogel loading ratio (Table I) have proved it. The data in Table I indeed demonstrate that most cavities in the scaffolds had been filled with the hydrogel, in particular for those with lower hydrogel concentration. This was especially prominent when the agar concentration was decreased from 2 to 1% (Table I, $p < 0.05$). There is no significant deference between the scaffold with 150 and 350 μm and in hydrogel loading rate ($p > 0.05$). But in practical operation, due to the poor fluidity of the hydrogel solution, the loading rate of the scaffold with a diameter of 150 μm varied in a relative larger range. In the next studies, the PLLA scaffold with larger pore size and the lower hydrogel concentration were chosen to ensure the sufficient filling of the pores.

It has been known that the scaffolds for tissue engineering must have the required mechanical integrity to maintain their predesigned micro- and macro-structures. They should also bear a certain load before new tissue formation. Some hybrid sponges comprising biomacromolecules (fibrin gel,
alginate and collagen etc) and polymer meshes have been utilized in tissue engineering and shown good cytocompatibility.29,35,36 In our study a “hard” scaffold with a porous microstructure was used instead of polymer meshes, which can provide much stronger mechanical strength. It is worth noting that the mechanical performance in hydrated state is extremely important because the scaffolds are used exclusively in contact with culture medium in vitro or body fluid in vivo. Unusually, we found the scaffold modulus in wet state was high than that of dry state (Figure 3). We attribute this modulus increase to the reinforcement effect of the water retained in the scaffold. According to the microstruc-

Figure 9. Hematoxylin and eosin staining of implants: (a) agar-filled scaffold, (b) the control scaffold. Chondrocytes/agar/PLLA scaffold construct after in vivo cultured for 4 weeks: (c) Masson’s Trichrome staining, (d) Immunohistochemical staining of collagen type II, (e) Alcian Blue staining of GAG. Original magnification ×100. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]
The cells escaping from the scaffold to the medium is an inevitable problem in the process of cell seeding. In this case, the chondrocytes were entrapped and stabilized within the agar/PLLA scaffold shortly after gelation by quenching from 40 to 25°C. Hence, this strategy can largely avoid cell loss which is frequently encountered for traditional cells/scaffold construct, that is a large amount of cells move from the scaffold to the culture medium before they adhere onto the scaffold. This advantage is clearly illustrated in Figure 4, where significant larger amount of chondrocytes was retained within the cells/agar/scaffold/construct than that within the cells/scaffold construct (control). The strategy may thus improve cell seeding efficiency which is crucial for tissue construction.

Another important feature of this construct is that the construct can keep the chondrocytes in normal round shape [Figures 5 and 6], and promote the cells viability, proliferation (Table II) by mimic the native growth environment of chondrocytes. In native cartilage the chondrocytes show normally round shape and are surrounded by a cross-linked matrix just like hydrogel. In this case, the agar gel served as an artificial ECM to embed and support chondrocytes just like their native growth environment. Therefore, the biomechanic structure of the hydrogel-filled scaffold is expected to keep the chondrocytes from dedifferentiation and to increase the cell performance. Furthermore, the gel structure would prevent the cell secretion, such as GAG, from losing into the medium during the culture.29 Higher cell secretion content, such as GAG and collagen were found in the cells/agar/scaffold construct compared with the control sample (Table II). Besides more favorable growth environment of the chondrocytes, the biomacromolecules diffusion resistances created by the agar gel network would also be one of the reasons.

The constructs could be simply “upgraded” by mixing with water soluble biomacromolecules or growth factors, such as gelatin, bFGF, TGF-β1, in the gel solution to enhance the chondrocytes performance. As a demonstration, gelatin was added into the agar/PLLA system which then formed a composite hydrogels. Since the gelatin is basically denatured collagen and presumably retains informational signals such as the Arg-Gly-Asp (RGD) sequence, incorporation of gelatin may also improve the chondrocytes’ performance. More chondrocytes with high cell viability and secretion level were found in the cells/agar/gelatin/scaffold constructs than those of cells/agar/scaffold constructs and the control sample (Figures 5 and 7, and Table I). The results demonstrate that the hydrogel filling strategy can indeed more effectively accelerate the chondrocyte growth and maintain the normal cell functions, in particular when gelatin is further incorporated. As aforementioned, gelatin contains some peptide sequences like RGD which are beneficial of attachment of those anchorage-dependent cells such as chondrocytes.37 Hence, this construct would facilitate the entrapment of soluble factors and create a suitable environment for cartilage regeneration.

To examine the cartilage regeneration behavior in vivo, the cells/hydrogel/scaffold (experimental) construct was implanted into nude mice. Here, the agar filled PLLA scaffold was adopted although the agar and gelatin filled scaffold has shown better in vitro performance for chondrocyte proliferation and differentiation, so that the interference of gelatin to collagen detection can be avoided. After 30d implantation, the cells/agar/scaffold constructs became cartilage-like in gross view (Figure 8). Histological examination shown the chondrocytes in the cells/agar/scaffold constructs were in the native round shape and surrounded by GAG and collagen type II [Figure 9(a,c,d,f)], while the cells in the control sample were rather small, fibroblast-like, and no evident the basophilic mucus and proteoglycan were found around the cells. These images revealed that the morphology of the chondrocytes and the expression of the cartilaginous matrices in the experimental construct were similar to those in native cartilage. The hydrogel filling strategy is more effective to regenerate normal cartilage compared with that of the traditional “hard” scaffold, by which cartilage with properties close to natural one can be formed. Together with the results of Figures 5 and 6, Tables II and III, one can thus conclude that the hydrogel-filled scaffold can provide a more friendly environment for chondrocytes to maintain their normal proliferation, differentiation and metabolism properties.

**CONCLUSIONS**

The cells, hydrogel and PLLA scaffolds are assembled together to mimic the native environments of cartilage ECM. By combining the advantages of “hard” and “soft” scaffolds, cells/agar/scaffold construct with good cytocompatibility and mechanical properties suitable for surgical manipulation and cartilage regeneration is obtained. The “hard” PLLA porous scaffold provides enough mechanical strength for shape maintenance and operation, while the “soft” hydrogel serves as a matrix surrounding the chondrocytes just like native ECM, resulting in higher cell viability and collagen and GAG secretions than that of PLLA-cell

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**TABLE III. Collagen and GAG Contents in the PLLA Scaffold (Pore Diameter 350 μm)/Agar/Chondrocyte Constructs Cultured In Vivo for 4 Weeks**

<table>
<thead>
<tr>
<th>Cells/Scaffold Construct</th>
<th>Cells/Agar/Scaffold Construct</th>
<th>Native Cartilage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collagen (wt %)</td>
<td>4.65 ± 0.1</td>
<td>6.81 ± 0.2</td>
</tr>
<tr>
<td>GAG (wt %)</td>
<td>1.9 ± 0.03</td>
<td>3.0 ± 0.1</td>
</tr>
</tbody>
</table>

Differences between different constructs are significant (p < 0.05) except for the collagen content of the scaffold/agar/chondrocyte construct and native cartilage.
construct. The cell performance can be further improved by incorporating gelatin in a very convenient mixing way. The results in vivo record a consistent tendency with the experiments in vitro. After implanted in the subcutaneous dorsum of the nude mice for 4 weeks, the chondrocytes/agar/scaffold constructs maintain their original rectangular shapes and display cartilage-like gross view. Histological examination of these specimens show that new cartilages are regenerated and a large quantity of collagen and GAG are determined. The results prove that the hydrogel filling strategy is more effective to regenerate normal cartilage compared with that of the traditional “hard” scaffold, by which cartilage with properties close to natural one can be formed. Moreover, the macroscopic shape of the construct and the regenerated cartilage is preserved. These features endow the present strategy with great advantages in cartilage tissue engineering.

REFERENCES


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