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In vitro engineering of human ear-shaped cartilage assisted with CAD/CAM technology

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ABSTRACT

Due to the lack of appropriate scaffolds, the *in vitro* engineering of cartilage tissue with a sophisticated structure, such as a human ear, remains a great challenge. Although polyglycolic acid (PGA) has become one of the most successful scaffolds for cartilage regeneration, how to overcome its limitations in achieving desirable mechanical strength and accurate control over shape remains an unsolved problem. In this study, the mechanical strength of PGA scaffold was enhanced by coating with polylactic acid (PLA). The content of PLA was optimized by balancing the scaffold's biocompatibility and mechanical strength. The PLA/PGA scaffold was then fabricated into a human ear-shape mirror-symmetrical to a normal ear by pressing the scaffold in the ear negative molds, which were fabricated by the computer aided design and manufacturing (CAD/CAM) technique according to the CT scan data from the normal ear. The ear-shaped scaffold reached a similarity level of over 97% compared to the positive ear mold by the shape analysis using a 3D laser scan system. Most importantly, after chondrocyte seeding, the constructs largely retained the original shape during culture with a similarity level of over 84%. Furthermore, the constructs formed ear-shaped cartilage-like tissues at 12 weeks, which revealed a tissue structure with abundant cartilage extracellular matrices and mature lacuna. Additionally, the ear-shaped cartilage at 12 weeks also exhibited fine elasticity and good mechanical strength. These results may provide a useful strategy for reconstructing cartilage tissue with complicated shapes such as a human ear by an *in vitro* engineering approach.

1. Introduction

Although tissue engineering is a promising method for repair and reconstruction of cartilage defects [1,2], engineering cartilage with a delicate three dimensional (3D) structure, such as a human ear, remains a great challenge in this field [3–5]. Since in 1997 Cao et al. engineered the cartilage with a shape of human auricle in a nude mouse model [6], many researchers have tried to explore further developments of this tissue engineering system, but few of them have succeeded in *in vitro* regeneration of a cartilage construct with a complete and anatomically refined auricle structure [7–13].

One major reason leading to the failure of *in vitro* engineering a cartilage construct with sufficient control over shape is the lack of appropriate scaffolds [3,4]. The optimal scaffold used for engineering a cartilage construct with accurate designed shapes should possess at least three characteristics: good biocompatibility for cartilage formation, ease of being processed into a specific shape, and sufficient mechanical strength for retaining the pre-designed shape during chondrogenesis [3–5]. Polyglycolic acid (PGA) has proven to be one of the most successful scaffolds for cartilage regeneration [14–16]. Cartilage engineered with the PGA scaffold has structure and composition similar to the native tissue, as demonstrated by histological analysis and cartilage specific matrices [17–19]. However, the most widely used form of PGA material in cartilage engineering is unwoven fiber mesh, which is difficult to be initially prepared into a complicated 3D structure and would most likely fail to maintain its original architecture during subsequent *in vitro* chondrogenesis due to insufficient mechanical support [17,20–22].

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To overcome these problems, two crucial issues should be addressed. First, the PGA-based scaffold should be prefabricated into the exact shape of a human ear. Second, the mechanical strength of the above-mentioned scaffold should be further enhanced so that it can retain the pre-designed shape during *in vitro* chondrogenesis.

In order to meet these requirements, in the current study, a computer aided design and manufacturing (CAD/CAM) technique was employed to fabricate a set of negative molds, which was then used to press the PGA fibers into the pre-designed ear structure. Furthermore, the mechanical strength of the scaffold was enhanced by coating the PGA fibers with an optimized amount of PLA. Then, the feasibility of engineering a shape controllable ear cartilage *in vitro* was explored by seeding chondrocytes into the optimized scaffolds. In addition, the exactness of the shape of the ear graft was quantitively evaluated by a 3D laser scanning system.

2. Materials and methods

2.1. Preparation of scaffolds with different PLA contents

PLA/PGA scaffolds were prepared similarly to the previously established methods [19]. Briefly, 40 mg of unwoven PGA fibers (provided by Dong Hua University, Shanghai, China) were compressed into a cylinder shape of 13 mm in diameter and 1.5 mm in thickness. A solution of 0.3% PLA (Sigma, St. Louis, MO, USA) in dichloromethane was evenly dropped onto the PGA scaffold, dried in a 65 °C oven, and weighed. The PLA mass ratio was calculated according to the formula: PLA% = (final mass-original mass)/final mass × 100%. The above procedures were repeated until the predetermined PLA mass ratios of 0%, 10%, 20% and 30% were achieved.

2.2. Mechanical analysis of the scaffolds

The mechanical properties of the scaffolds were analyzed by a biomechanical analyzer (Instron-5542, Canton, MA, USA). Similar to previously reported methods [23], the scaffold disks were compressed at a constant compressive strain rate of 0.5 mm/min until a maximum of 10% total strain was reached. The maximum compressive force and Young's modulus were determined from the stress–strain curve.

2.3. Biocompatibility evaluation of the scaffolds

2.3.1. Cell seeding

Chondrocytes were isolated from the articular cartilage of newborn swine (2–3 weeks old) as previously described [24]. The harvested chondrocytes were adjusted to a final concentration of 50×10^6 cells/mL, and a 200 μL cell suspension was piped onto each scaffold. The cell-scaffold constructs were then incubated for 5 h at 37 °C with 95% humidity and 5% CO_2 to allow for complete adhesion of the cells to the scaffolds. Then, the constructs were covered by pre-warmed culture medium and cultured under the same conditions.

2.3.2. Cell adhesion

After 24 h of incubation, the cell-scaffold constructs were gently transferred into a new 6-well plate for subsequent culture to evaluate cartilage formation. The remaining cells were collected and counted. The cell seeding efficiencies of the scaffolds with different PLA contents were calculated based on the formula: (total cell number – remaining cell number)/total cell number × 100% [17].

2.3.3. Scanning electron microscopy (SEM)

The constructs were cultured *in vitro* and the attachment and matrix production of the cells on the scaffolds were examined by SEM (Philips XL-30, Amsterdam, Netherlands) after 2 weeks and 8 weeks [25].

2.3.4. Evaluation of cartilage formation

The constructs were harvested after 8 weeks of culture. The cartilage formation on different scaffolds was evaluated histologically by staining with hematoxylin and eosin (HE) and Safranin-O, and immunohistochemically with type II collagen [19].

2.4. Mold fabrication by CAD/CAM

A patient's normal ear was scanned by CT to obtain the geometric data. These data were further processed by a CAD system to generate the half-sized mirror image data (both positive and negative) of the normal ear, and the resultant data were input into a CAM system (Spectrum 510, Z Corporation) for the fabrication of the resin models by 3D printing. The negative mold was composed of two parts: the outer part and the inner part. In order to make the mold pressure-loadable, the outer part was replaced by a silicon rubber, which was molded according to the inner part of the resin negative mold [26].

2.5. Fabrication of ear-shaped scaffold

Two hundred milligrams of unwoven PGA fibers were pressed using the negative mold for over 12 h. A solution of 0.3% PLA (Sigma, St. Louis, MO, USA) in dichloromethane was evenly dropped onto the PGA scaffold, dried in a 65 °C oven, weighed, and pressed again with the negative mold. This procedure was repeated until the final PLA mass ratio of 20% was reached. The edge of the scaffold was carefully trimmed according to the shape of the positive mold.

2.6. Three-dimensional laser surface scanning

A 3D laser scanning system was used for the shape analysis [27]. The surface image data were collected from both the positive mold and the ear-shaped scaffolds using a Konica Minolta Vivid 910 and Polygen Editing Tools version 2.21 (Konica Minolta, Tokyo, Japan). These data were further processed by Rapid Form 2006 (INUS, Seoul, South Korea) and HP xw6200 (Hewlett Packard, Shanghai, China). The resultant data obtained from the ear-shaped scaffolds were compared to those from the positive mold, which served as a standard. Variations in voxels smaller than 1 mm were considered similar, and the number of these similar voxels was divided by the number of total voxels to calculate the similarity level.

2.7. In vitro construction of ear-shaped cartilage

A 1 mL aliquot of chondrocyte suspension with a density of 50×10^6 cells/mL was seeded into the ear-shaped scaffold followed by incubating for 5 h, according to the cell seeding procedures described above. Then, the construct was gently transferred into a 50 mL centrifuge tube for subsequent culture. The culture medium was changed every other day. The constructs were harvested at 4 weeks, 8 weeks and 12 weeks for evaluation of shape exactness and cartilage specific histology.

2.8. Statistical analysis

The differences of cell seeding efficiencies ($n = 6$), Young's moduli ($n = 6$), and maximum compressive loadings ($n = 6$) among the four PLA content groups were analyzed using the Student's *t*-test. A *p*-value less than 0.05 was considered statistically significant.

3. Results

3.1. Mechanical analysis of different scaffolds

The mechanical properties of the scaffolds were analyzed to evaluate the effects of PLA coating with different amount on the mechanical strength. As shown in Fig. 1, all the scaffolds had regular cylinder shapes with the same diameter of 13 mm (Fig. 1A–D). No obvious differences in appearance were observed among the PLA/PGA scaffolds with different PLA amounts (Fig. 1B–D). As expected, the pure PGA group (0% PLA group) showed a flat compressive stress–strain curve close to the *X* axis, indicating that pure PGA scaffolds had relatively low mechanical strength. With an increase in PLA content, the compressive stress–strain curves became steeper and more linear before the maximum loadings were reached (Fig. 1E), and the compressive moduli (Fig. 1F) as well as maximum loadings (Fig. 1G) also increased. Noticeably, there was a significant increase (over 4 folds) in both compressive moduli and maximum loading in scaffolds fabricated with 20% PLA compared to those with 10% PLA. Furthermore, the scaffold with 20% PLA reached a compressive modulus around 45 MPa (45.42 ± 10.52 MPa), which was similar to that of native adult human articular cartilage [19]. As expected, the 30% PLA group achieved the highest maximum loading and Young's modulus in all groups, although no significant difference was observed in Young's modulus between the 20% and 30% groups.

3.2. Evaluation of the biocompatibility of the scaffolds with different PLA contents

Cell seeding efficiencies, SEM, and histological examination were performed to evaluate the influence of PLA contents on cell compatibility of the scaffolds and on final cartilage formation. The