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3D-printed scaffolds with calcified layer for osteochondral tissue engineering

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Treating full-layer injury of bone and cartilage is currently a significant challenge in orthopedic trauma repair. Joint damage typically includes chondral defects, and the underlying subchondral defect sites are difficult to repair. Tissue engineering technology could potentially be used to treat such injuries; however, results to date been unsatisfactory. The aim of this study was to design a multilayer composite scaffold containing cartilage, bone, and calcified layers to simulate physiological full-thickness bone-cartilage structure. The cartilage layer was created using an improved temperature-gradient thermally induced crystallization technology. The bone and calcified layers were synthesized using 3D printing technology. We examined the scaffold by using scanning electron microscope (SEM), X-ray diffraction (XRD), fluorescence staining, and micro computed tomography (Micro-CT), and observed clearly oriented structures in the cartilage layer, overlapping structures in the bone scaffold, and a compressed calcified layer. Biomechanical performance testing showed that the scaffolds were significantly stronger than scaffolds without a calcified layer (traditional scaffolds) in maximum tensile strength and maximum shear strength ($P < 0.05$). After inoculating cells onto the scaffolds, we observed similar cell adherence and proliferation to that observed in traditional scaffolds, likely because of the high porosity of the whole scaffold. Our scaffolds could be used in bone and cartilage full-thickness injury repair methods, as well as applications in the field of tissue engineering.

[**Key words:** 3D-printed scaffolds; Bone; Cartilage; Tissue engineering; 3D printing]

Osteochondral joint trauma caused by disease or various complex physical injuries could result in considerable damage and a poor prognosis, often resulting in disability or teratogenicity after surgery (1,2). Elderly patients typically require surgical treatment and could eventually experience considerable loss of movement. Hyaline cartilage lesions, in particular, can cause severe pain (3). Treatment methods are often selected based on factors such as age, gender, general situation, location of injuries, and diameter and depth of damage (4,5). Many patients usually need arthroscopic surgery or even joint replacement. Although autologous osteochondral transplantation is now recognized as the gold standard for clinical treatment, it has many inherent limitations, including limited donors, difficulty in obtaining matching transplant shape, and the influence of healthy tissue surrounding the transplant.

Tissue engineering (TE) strategies could have significant advantages over traditional clinical treatment methods. Tissue engineering systems include seed cells, biological materials, and growth factors, which could promote regeneration of native host cells (6,7). There are two main types of scaffolds used as delivery vehicles: (i) cell-free scaffolds, wherein the host cells infiltrate into the surrounding tissue and initiate tissue repair, and (ii) vaccinated cell scaffolds, wherein cells are harvested from patients and cultivated on scaffolds in vitro before implantation. Scaffold properties, including porosity, pore size, and substrate rigidity factors, can affect cell infiltration and attachment. Some scaffolds are produced using synthetic materials such as polylactic acid (PLA), polyglycolic acid (PGA), Polylactic-co-glycolic acid (PLGA), β-tricalcium phosphate (β-TCP), and Hydroxyapatite (HA) (8,9). Others are composed of natural materials, including type I collagen, type II collagen, fibrous protein, hyaluronic acid, and agarose (10). Both types of scaffolds have been widely used to repair bone-cartilage injuries. Of these, HA, PLGA, and type II collagen have specific advantages, including potential support of cell adhesion, proliferation, and migration. The extracted bovine cartilage matrix contains collagen subtypes of II, VI, IX, X and XI. Type II collagen is 90–95% of the total amount of collagen. Thus, we used a mixture of HA, PLGA, and extracted bovine cartilage matrix to produce scaffolds in this study, in order to optimize biological activity. Moreover, our scaffold had the added advantage of minimal emission of harmful substances.

One of the major challenges in choosing biomaterials for osteochondral scaffolds is to mimic natural tissue structure adequately (11,12). Articular cartilage is a highly organized tissue, which provides a low-friction, wear-resistant bearing surface and has anisotropic mechanical properties. Therefore, the extracellular matrices (ECMs) of articular cartilage have depth-dependent structural arrangements. Articular cartilage is comprised of two main zones from a functional perspective: the oriented and calcified cartilage zones. Our aim was to create one completely bionic

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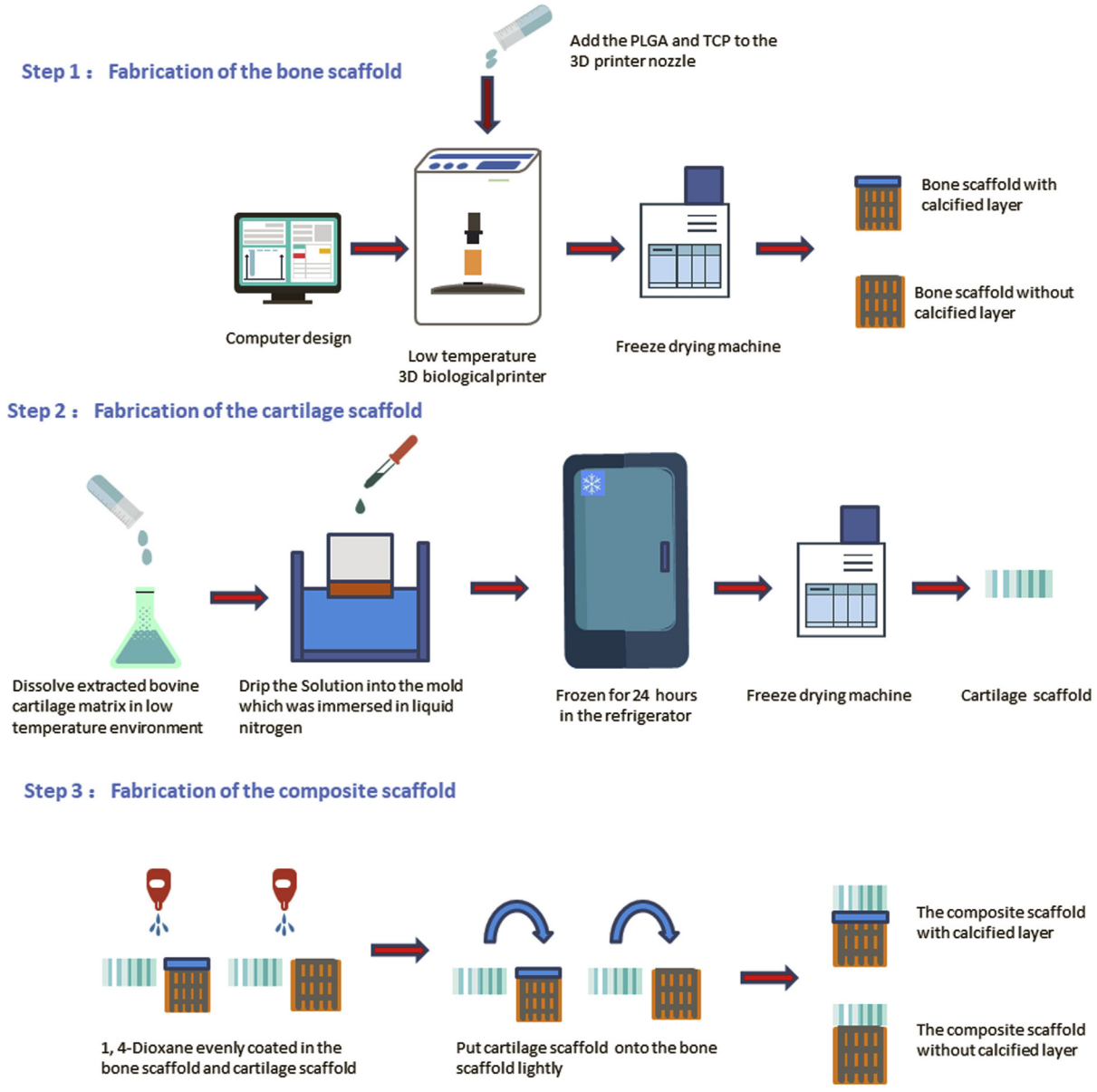

FIG. 1. Schematic representation of the multilayer scaffold fabrication process showing the three main steps involved in the fabrication of the scaffolds.

cartilage and bone scaffold. In this study, we attempted to produce a multi-layered scaffold, which includes an oriented cartilage layer, cartilage calcification layer, and biological bone layer, wherein cells recruited from the bone marrow could attach, proliferate, and infiltrate satisfactorily. The calcified cartilage layer would not only increase the mechanical strength, but could also separate the chondrocyte and bone cell microenvironments, thus promoting the growth of cells in their respective areas.

This study further aimed to test the mechanical properties of the complicated micro-architectural structure of the multilayer scaffold. We evaluated the distribution, proliferation and morphology of cells in the upper zones and lower zones, in order to understand the in vitro effects of the physical properties and 3D internal structures of different materials.

MATERIALS AND METHODS

Bone zone/calcified layer fabrication PLGA and β-TCP were purchased from Sigma Aldrich (St. Louis, MO, USA). We designed and developed a low-temperature 3-D biological printer (Tsinghua university, Beijing, China) in order to build the bone/calcified layer of our scaffold. First, we designed a model on the computer. We then dissolved PLGA in 1, 4—dioxane to obtain a 3% solution, and added 1% β-TCP to obtain a turbid solution at room temperature (25°C). The solution was placed on a magnetic stirrer until it dissolved adequately, and injected into the container of the 3D printer; container temperature was −30°C. The computer was adjusted to control the movement of the container (2 mm/s) in order to ensure precise distance between layers (300 ± 6 μm). The first scaffold layer printed was the calcified layer. The liquid from the container flowed into the nozzle and was ejected onto the workbench surface at −30°C, so that it immediately froze into a solid. The nozzle moved continuously and closely, and there was no space between two trajectories. Next, we used computer software (Tsinghua university, Beijing, China) to create the remaining three overlapping layers. The nozzle is controlled by the computer, which ensures that the nozzle moves accurately in accordance with the design trajectory. Under the effect of the pressure between each layer, the distance between two layers was shortened to about 200 ± 8 μm. After 2 h, stereoscopic 3D bone scaffolds were obtained, which included the calcified layer. These scaffolds were lyophilized for 12 h in order to sublimate the 1,4-dioxane. Conventional scaffolds without the calcified layer were produced using the same method. However, the first layer was not produced.

Directional cartilage layer fabrication We collected fresh bovine limbs and sliced out the articular cartilage under aseptic conditions. We then used cell-free technology and freeze-drying to obtain spongiform substrate. Cartilage matrix

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