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Carbon dioxide-assisted bioassembly of cell-loaded scaffolds from polymeric porous microspheres

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ABSTRACT

A cell-friendly strategy based on the subcritical carbon dioxide (Sub-CO_2) sintering was developed for convenient single-step bioassembly of scaffolds from cellularized poly(lactic-co-glycolic acid) (PLGA) porous microspheres. The method has provided a versatile strategy to utilize the hierarchical voids of the scaffold, which allowed seeded cells to proliferate for 14 days (cartilage cells) and 21 days (fibroblasts), respectively. Furthermore, confocal laser scanning microscopy (CLSM) and scanning electron microscopy (SEM) images indicated that the cartilage cells favored the surface of the porous microspheres, while the fibroblasts tended to grow into the porous microspheres and attach onto the walls of the inner pores. This modular strategy provides a convenient way to seed cells prior to bulk scaffold fabrication as the Sub-CO_2 processing is benign to cells, which will potentially find widespread applications in tissue engineering and regenerative medicine.

1. Introduction

Traditional tissue engineering strategies typically employ a 'top-down' approach, in which cells are seeded onto a pre-formed three-dimensional (3D) polymeric scaffold [1]. The classical methods, including gas foaming, particle leaching, phase separation, and electrospinning, could fabricate scaffolds with open pores and interconnected bulks [2]. However, in these approaches most of the cells can only attach to the surface of the scaffolds [3]. In addition, methods such as centrifuge seeding [4] and orbital shaker seeding [5] have been introduced to improve cell penetration, the cell distribution in the interior still largely depends on the microstructures of scaffolds, which significantly impact cell distribution and subsequent tissue formation [6,7].

In contrast, a modular tissue engineering strategy is devoted to design a biomimetic microstructure, through assembling cells, biomolecules, and modular unit towards an integrated macrotissue [1]. Microspheres make versatile carriers and they have demonstrated a variety of applications in the biomedicine, including drug delivery [8–10] and use as scaffolds for tissue engineering [11,12]. The utilization of microspheres in scaffold fabrication has promoted the precision of the modular assembling strategy and offers the advantages of interconnecting porous structures, with the potential of incorporating spatiotemporal controlled release of bioactive agents [13,14].

Gas sintering [15,16], heat sintering [17–19] and solvent sintering [20,21] are conventional methods of producing microsphere-based scaffold assemblies. They all sinter microspheres together to form a cohesive construct. In addition, various improvements have been made in the process of fabricating sintered microsphere scaffolds, such as entrapping drugs into composite materials [22], producing a microsphere-based scaffold with a porous rough surface to enhance cell attachment [23], as well as systematic investigation of the properties of raw materials, including the molecular weight, co-polymer ratio, and microsphere size to match the degradation kinetics of scaffolds. However, in the area of microsphere-based scaffolds, precise management of the sintering process for the cell-loaded microspheres have not yet been reported.

Recently, the inexpensive and low-toxicity CO_2 has been introduced as an excellent 'green solvent' technology for scaffold fabrication [24–26]. In terms of sintering, the utility of CO_2 avoids

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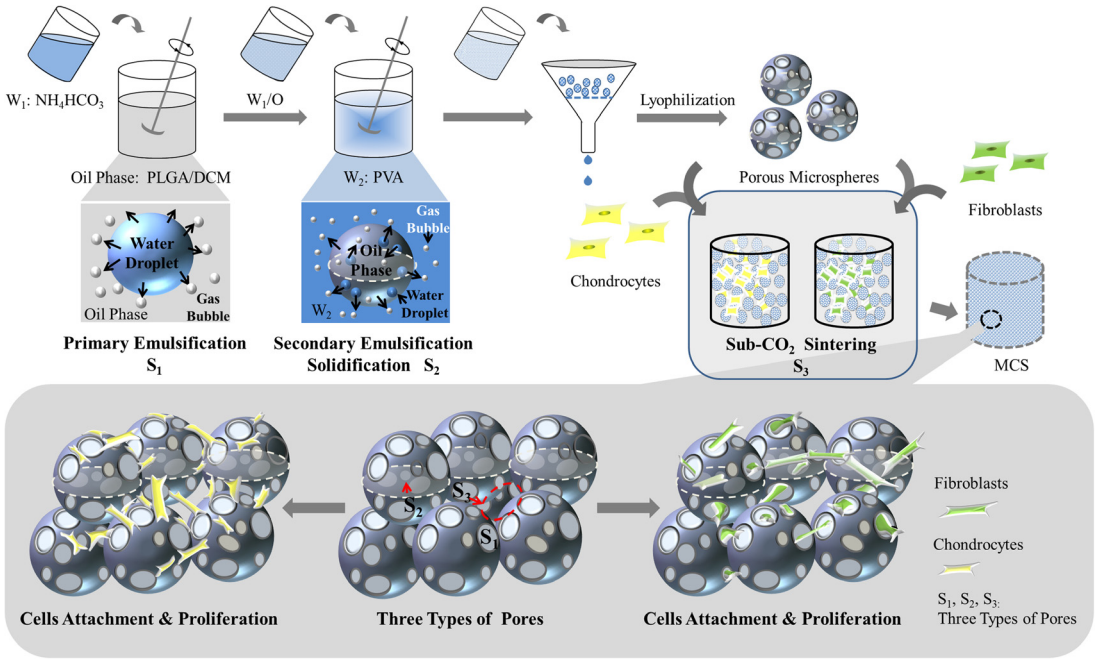

Fig. 1. The schematic diagram showing carbon dioxide-assisted bioassembly of MCSs. Primary Emulsification: Ammonium bicarbonate of W_1 (inner water phase) decomposes and forms gas bubbles (NH_3 and CO_2); this hinders the separation of water phase from oil phase, and then results a homogeneous dispersion in the oil phase, thus forming the precise microstructure S1. Secondary Emulsification: the primary emulsification solution will be dispersed homogeneously in W_2 (outer water phase), followed by the escape of gas bubbles from the inner water phase into the viscoelastic PLGA walls generated by the gradual removal of dichloromethane (DCM), which leads to formation of microstructure S2. Lyophilization: removes the water phase thoroughly. Sub-CO_2 sintering: low-pressure CO_2 plasticizes the surface of the microspheres and bonds neighboring microspheres, thus forming the microstructure S3 (The void between neighboring microspheres).

exposing the biomolecules to elevated temperatures or organic solvents, which may lead to inactivation of cells [27,28], protein drugs, or bioactive agents. In addition, Sub-CO_2 depresses the glass transition temperature (T_g) of the material surfaces and broadens the rubbery layer at the same temperature without deforming the morphology of the polymeric scaffold [29], where as CO_2 sintering of few polymers (such as PCL) results in melting point depletion [26]. As such, cells loaded in the scaffolds could remain viable and functional when CO_2 is used as the sintering agent [28,30,31]. The dense gas makes the surface materials of the microspheres swell and fuse together.

Inspired by these facts, this study reports the development of a cell-friendly strategy based on the Sub-CO_2 sintering technology, for single-step fabrication of a porous microsphere-based cell-loaded scaffolds (MCS). In a typical procedure, porous microspheres are first fabricated using an emulsification approach, followed by their volumetric packing in the presence of cells, where the junctions of the adjacent microspheres are subsequently sintered by Sub-CO_2 treatment (Fig. 1). Cell distribution, proliferation, and activity within the scaffolds were explored to prove the benign sintering process of the embedded cells. In particular, this strategy not only achieved *in situ* assembly of the cell-carrying porous microspheres into 3D scaffolds without obvious deformation of the microspheres, but also promoted the homogeneous distribution of the cells inside the scaffolds through the single-step sintering process.

2. Materials and methods

2.1. Materials

Poly(lactic-co-glycolic acid) (PLGA, lactic/glycolic acid 50:50; $M_w \sim 40{,}000$ Da) was purchased from the Jinan Daigang Biological Co., Ltd. (Jinan, China). Ammonium bicarbonate (AB) and dichloromethane (DCM, 99.8% purity) were obtained from the Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Polyvinyl alcohol (PVA; $M_w \sim 22{,}000$ Da) was supplied by Yikang Science and Technology Inc. (Beijing, China) and CO_2 of 99.9% purity was purchased from the Rihong Air Products Co., Ltd. (Xiamen, China).

2.2. Cell culture

The rat chondrocyte cell line RCJ3.1C5.18 (C5.18) and NCTC clone 929 (L929) cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco), with 10% fetal bovine serum (Hyclone), 100 U mL^{-1} of penicillin (Gibco), and 100 μg mL^{-1} of streptomycin (Gibco) at 37 °C in a humidified incubator with 5% CO_2.

2.3. Cytotoxicity study of Sub-CO_2 and fabrication steps

The effect of the Sub-CO_2 process on cytomembrane was verified. A cytosolic lactate dehydrogenase (LDH) assay was performed to assess the cell integrity. C5.18 and L929 cells were separately suspended in DMEM and treated with Sub-CO_2 at different exposure time, pressure, and depressurizing rate. The cells were then centrifuged at 1000 rpm for 3 min, and the supernatant was analyzed for the release of LDH using an LDH kit (Jiancheng Biotech. Inc., Nanjing, China). As a control, the supernatant of cells without Sub-CO_2 treatment was detected. Absorbance at λ 450 nm was measured.

The Cell Counting Kit-8 (CCK-8) assay was then used to measure the cytotoxicity of Sub-CO_2. C5.18 and L929 cells were separately suspended in DMEM and treated with Sub-CO_2 at 25 °C, 2 MPa for 10 min. After treatment, cells were centrifuged at 1000 rpm for 3 min and the precipitate (cells) were cultured with fresh DMEM for 14 days and analyzed by the CCK-8 assay at different time points. The group without Sub-CO_2 treatment was used as control.