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Controlling oxygen release from hollow microparticles for prolonged cell survival under hypoxic environment

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ABSTRACT

The survival of cells in the 3D scaffold until the ingrowth of blood vessels is one of the most important challenges in tissue engineering for producing a clinically relevant volume of tissue. In this study, perfluorooctane emulsion (oxygen carrier)-loaded hollow microparticles (PFO-HPs) were prepared as a scaffolding system which can allow timely release of oxygen to cells adhered on the HPs to prevent cell necrosis in a hypoxic environment (inherently created in tissue engineered 3D constructs) until new blood vessels are formed in the 3D cell construct, and thus may produce appropriate tissues/organs with a clinically relevant volume. In the *in vitro* cell culture and the *in vivo* animal study, it was observed that the cells initially seeded on the PFO-HPs remained alive for approximately 10 days in a hypoxic environment (*in vitro*), and the cells were also found throughout the implanted whole matrix without a necrotic center until the infiltration of blood vessels (at 14 days after implantation; *in vivo*), probably due to the sufficient release of oxygen from the PFO-HPs for an adequate time period. Based on these results, the cell-based PFO-HPs can be a promising system to produce a clinically applicable large tissue mass.

1. Introduction

The survival and functional maintenance of cells in the 3D matrix (i.e., scaffold) until the ingrowth and maturation of blood vessels is the most important challenge in tissue engineering for producing a clinically relevant volume of tissues/organs [1]. Although innovative tissue engineered tissues/organs such as the skin, trachea, and bladder have been reported [2], their limited dimensions caused by slow vascularization in the scaffold [3,4] and limited oxygen diffusion into the non-vascularized scaffold before blood vessel formation, which lead to insufficient oxygen supply to cells [5], are significant hurdles for clinical application. The oxygen dissolved in the aqueous solution (cell culture medium or body fluid) can be diffused limitedly (100–200 μm) to cells seeded on the scaffold; therefore, the cells should be located within the critical distance from oxygen sources to prevent hypoxia and necrosis [6]. It is well known that the central region of the engineered tissues with a volume larger than 1 cm^3 is usually in a hypoxic environment, and thus imperfect tissues with a necrotic center and viable cells only at the periphery are produced [1,7]. To overcome the inherent limitation of tissue engineering, several strategies including the use of oxygen carriers (perfluorocarbons, fluorinated-zeolite [8–10]) and angiogenic factors (vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), endothelial cells, and VEGF cDNA-transfected stem cells [11–15]) have been incorporated into the scaffolds. Although promising results in a relatively small volume of tissue have been reported, inadequate oxygen delivery to the cells located in the center of the scaffold caused by the heterogeneous distribution [in particular, perfluorocarbons located at the bottom of the scaffold due to their high density (1.5–2.0 folds higher than that of water)] [16] and/or limited loading amount of oxygen carriers [10] and slow rate of blood vessel growth in the scaffold in spite of the use of angiogenic factors [17] can be the obstacles in producing a clinically applicable large tissue mass. More recently, oxygen-producing material-loaded scaffold systems, which can produce oxygen by decomposition or chemical reaction of peroxides incorporated into the scaffolds, and thus can provide an appropriate oxygen environment

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for cell survival in the overall scaffold regardless of the dimensions, have been developed. Hydrogen peroxide [18,19], sodium percarbonate [20,21], calcium peroxide [22–24], and pyridine endoperoxides [25] are utilized as oxygen-producing materials. However, uncontrolled oxygen release and cytotoxicity of the peroxides themselves and their by-products during oxygen production [26] are also issues. The use of cells with downregulated metabolic activity which reduces oxygen consumption of cells and thus prolongs cell survival in a hypoxic environment until new blood vessel formation is also proposed as a tool to overcome the huge barrier of tissue engineering [1]. Based on these research findings, we hypothesized that if a scaffolding system that provides timely and uniform oxygen delivery to the cells close to the surrounding oxygen concentration for a sufficient time period (until blood vessel ingrowth into the scaffold from the host system) could be developed, it may be a promising strategy to produce tissue engineered tissues/organs with a clinically relevant volume.

The main aims of this study are i) to develop hollow microparticles (HPs) containing oxygen carrier as a scaffolding system which can allow uniform distribution of oxygen carrier in each HP and timely supply of oxygen to cells adhered on the HPs in order to prevent cell necrosis in a hypoxic environment until blood vessel infiltration into the 3D cell construct, and thus produce tissues/organs with an appropriate volume for clinical application, and ii) to perform a feasibility study of the oxygen carrier-loaded HPs as a scaffolding system for 3D tissue formation. To achieve this purpose, we prepared perfluorooctane emulsion (PFO; as an oxygen carrier)-loaded (entrapped) hollow microparticles (PFO-HPs) using the W/O/W emulsion solvent evaporation method (for HP) and infusion of PFO into the HP. The oxygen carrier-loaded HP is expected to have a stable location of oxygen carrier in the HP without flow down due to its high density, and to provide controlled release of oxygen to the surrounding cells through the shell membrane with micropores, and thus avoid cell necrosis even in a low oxygen environment. Therefore, the PFO-HP may be a simple and practical scaffolding system for reconstruction of engineered 3D tissues/organs. Fig. 1 demonstrates the schematic diagrams of the PFO-HP (oxygen supply) and PBS-loaded HP (PBS-HP, non-supply of oxygen), and their possible phenomena during *in vitro* cell culture (in hypoxia) and *in vivo* 3D tissue formation. Cell proliferation and oxygen concentration in the PFO-HPs and PBS-HPs (in hypoxia, 1% O_2) were compared (*in vitro*). Live cell distribution and blood vessel formation in the implanted cell/HP matrix were also investigated using an animal model (nude mouse, subcutaneous injection).

2. Materials and methods

2.1. Materials

Polycaprolactone (PCL; Mw 70,000–90,000 Da; Sigma–Aldrich, USA), Pluronic F127 (Mw 12,500 Da; Sigma–Aldrich), and Poly (vinyl alcohol) (PVA; Mw 85,000–124,000 Da; hydrolysis, 87–89%; Sigma–Aldrich) were used to fabricate PCL HPs. Perfluorooctane (PFO), 1,2-Diacyl-sn-glycero-3-phosphocholine (egg yolk phospholipid; EYP), Pluronic F68 (Mw 8,400 Da) were purchased from Sigma–Aldrich to prepare the PFO emulsion. All other chemicals were of analytical grade and were used as received. Water was purified using a Milli-Q purification system (Millipore Co., USA). For *in vitro* cell culture and *in vivo* animal studies, PCL HPs were sterilized by ethylene oxide (EO) and PFO emulsion was autoclaved.

2.2. Preparation of PCL hollow microparticles

PCL HPs were fabricated using a modified W/O/W emulsion solvent evaporation method [27]. PCL pellets were dissolved in ethyl acetate (EA) to 5 wt% at room temperature (4 mL, oil phase), and 1 wt% Pluronic F127 aqueous solution (1 mL, internal water phase) was added to the PCL solution with stirring (400 rpm) to emulsify the solution. The emulsion solution was directly poured into 0.5 wt% PVA aqueous solution (1 L) with gentle stirring (400 rpm) to re-emulsify the solution, and the droplets were solidified for 10 min. The solidified HPs were washed with excess water to remove EA, Pluronic F127, and PVA, and then the PCL HPs were separated in size ranges of 100~500 μm by wet microsieving using standard testing sieves (Chunggye Industrial Co., Korea), and were freeze dried. The morphologies of the PCL HPs were observed by a scanning electron microscope (Model JSM-6510, JEOL, Japan). The cross-section samples were prepared by cutting the PCL HPs after freezing (−80 °C) in Tissue Tek OCT compound (Miles Scientific, USA), washing with excess water, and freeze-drying.

2.3. Preparation of PFO emulsion-loaded PCL hollow microparticles

As the emulsifying agents for PFO, 20 wt% Pluronic F68 solution and 4 wt% EYP solution were prepared by dissolving in phosphate buffered saline (PBS, pH ~ 7.4), respectively. The PFO/Pluronic F68 solution/EYP solution mixture (6/2/2, v/v/v) was vigorously agitated using a probe type ultrasonic wave homogenizer (Branson Sonifier model 185, USA) to produce nano-sized emulsion droplets. Ultrasonication (30 s ON and 20 s OFF; 10 cycles) at a frequency of 18,000 Hz was directly applied to the mixture solution placed in warm water bath (60 °C). The average size of the prepared PFO emulsion droplets was measured by dynamic light scattering (Mastersizer 2000, Malvern, UK) at room temperature. For loading of the PFO emulsion into the PCL HPs, the prepared PFO emulsion (15 mL) was poured in a needle tip-stopped syringe (20 mL) filled with the PCL HPs (occupied volume, 5 mL). The emulsion was infiltrated into the PCL HPs through the shell membrane with a micropore under positive pressure when the syringe piston was pushed in. Then the PCL HPs were carefully washed with PBS to remove the unloaded PFO emulsion. As a control group, the PBS-loaded PCL HPs were also prepared using the same procedure described above. To evaluate whether the PFO emulsion can penetrate into the PCL HPs, the PFO emulsion was stained by Nile Red. The Nile Red-labeled emulsion in PCL HPs was visualized using a confocal laser scanning microscope (LSM 700, Carl Zeiss, Germany). The Nile Red-stained PFO emulsion was prepared by simple mixing of 1.5 mg/mL Nile Red solution (in acetone) and the PFO emulsion (1/500, v/v) for 10 min, and the un-reacted Nile Red was removed by dialysis using a dialysis membrane with a molecular weight cutoff of 3,500 Da (in PBS, 5 h; CelluSep®, Membrane Filtration Products, USA).

2.4. In vitro cell culture

To investigate cell survival in a hypoxic environment using the PBS-HPs and PFO-HPs, the murine calvaria pre-osteoblast (MC3T3-E1; CRL-2593, ATCC, USA) was used as the cell model. Before cell seeding on the HPs, the HPs (5 mL) placed in the 20 mL sample tube (SPL, Korea) were stored in an aseptic oxygen chamber ($7.5 \times 6.0 \times 4.0$ cm^3; O_2 flow rate, 5 mL/h) for 5 min to achieve oxygen saturation. Then, the oxygen-saturated HPs were immediately transferred to non-treated 24-well PS dishes (200 μL/well; Corning, USA). One milliliter of cell suspension [cell density, 1.0×10^6 cells/mL; α-MEM supplemented with 10% fetal bovine serum (Gibco, USA), 100 U/mL penicillin and 100 μg/mL streptomycin (Invitrogen, USA)] was seeded on the HPs, and shaken mildly under 50 rpm for 24 h for uniform cell adhesion on the HPs in a normal incubator (21% O_2, 5% CO_2, 37 °C). Then, the cell-seeded HPs were carefully transferred to new non-treated 24-well PS dishes, and the medium was added into each well (2 mL) and then cultured in a hypoxic incubator (1% O_2, 5% CO_2, 37 °C) for 14 days. The cell culture on the non-treated dishes may minimize migration of cells attached on the HPs to the dish surfaces. The culture medium was not exchanged during the cell culture period to prevent re-oxygenation of the medium, but the excess medium which can prevent cell starvation and pH drop (caused by cell metabolism) was used at the initial stage. The number of viable cells on the HPs after cell culture for 0, 1, 3, 5, 7, 10 and 14 days was estimated by a CCK-8 assay.

Oxygen concentration in the cell culture medium surrounding HPs during cell culture in a hypoxic incubator was measured using a portable dissolved oxygen meter (MonoLine Oxi 3410 IDS, WTW, Germany). Five milliliters of cell solution (cell density, 1.0×10^6 cells/mL) was seeded on the oxygen-saturated HPs (PBS-HP & PFO-HP; occupied volume, 1 mL) placed in a 20 mL sample tube (SPL) and shaken mildly under 50 rpm for 24 h in a normal incubator, and the medium was added into the tube (10 mL) and then cultured in the hypoxic incubator for 14 days. The oxygen concentration in the cell culture medium was measured (0, 1, 3, 5, 7, 10 and 14 days). After the oxygen concentration measurement at each time point, the specimens were discarded to prevent re-oxygenation of the medium. The oxygen concentrations in the medium stored in normal atmosphere and 5% CO_2 incubator were also determined.

The oxygen carrying capacity per HPs was also determined by the dissolved oxygen amount in PFO emulsion or PBS loaded in the HPs with unit volume. To this, the volume of PFO emulsion or PBS loaded in the 1 mL (occupied volume) HPs, and the dissolved oxygen concentration in the oxygen-saturated PFO emulsion or PBS (oxygen saturation by same procedure with *in vitro* cell culture) were measured ($n = 3$), respectively. The oxygen carrying capacity per 1 mL HPs was calculated as follows: Total loading amount of oxygen in HPs of 1 mL (μg) = dissolved oxygen concentration in PFO emulsion or PBS (μg/mL) x volume of PFO emulsion or PBS loaded in the 1 mL HPs (mL). The volume of PFO emulsion or PBS loaded in the 1 mL HPs was estimated by the reduced volume of PFO emulsion or PBS after the infiltration into the PCL HPs under positive pressure.

2.5. Quantitative real time polymerase chain reaction (real-time PCR) analysis

At the predetermined time intervals (14 days) of cell culture in each HP group, the RNA was extracted using an Qiagen RNeasy Mini kit (Qiagen, USA) according to