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Development of human-derived hemoglobin–albumin microspheres as oxygen carriers using Shirasu porous glass membrane emulsification

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Novel hemoglobin-based artificial oxygen carriers are necessary in tissue engineering. We developed human hemoglobin (hHb) and albumin (HSA)-based microspheres using Shirasu porous glass (SPG) membrane emulsification. The obtained microspheres had a uniform size with an average diameter of 15.1 μm measured by optical microscope, which is similar to the diameter of human red blood cells (7–8 μm). The loading amount of hHb in the microspheres was 20 wt%, which is similar to that of red blood cells (33 wt%). The hHb–HSA microspheres showed similar oxygen dissociation behavior and methemoglobin formation resistance to native hHb. Incubation with genetically engineered HeLa cells that shows hypoxia-responsive EGFP expression demonstrated efficient oxygen supply from the microspheres. Our study suggests the utility of hHb–HSA microspheres as oxygen carriers for tissue engineering with a low risk of infectious disease.

[**Key words:** Oxygen carrier; Hemoglobin; Microsphere; Shirasu porous glass membrane emulsification; Hypoxia-responsive cell; Tissue engineering]

Tissue engineering has attracted a lot of attention as a potential alternative or complementary solution for organ failure (1,2). When culturing three-dimensional (3D) tissues *in vitro*, limited oxygen supply is one of the most critical issues. Oxygen diffusion from culture media is often insufficient to compensate for oxygen consumption from tissues, leading to the generation of necrosis caused by hypoxia (3,4). To improve this oxygen supply, fabrication of a vascular network or microchannel in 3D tissues and perfusion of oxygen carriers through channels have been extensively studied (5–9). Recent studies have reported successful perfusion of blood containing red blood cells (RBCs) in 3D reconstructed tissues (6). However, handling of native blood is still difficult, preventing its practical application at the current stage. For example, perfusion of blood often causes hemolysis of RBCs, which potentially exhibits cytotoxicity.

As an alternative, use of hemoglobin (Hb)-based oxygen carriers (HBOCs) is expected to be promising for preventing hypoxia in regenerated tissues (10–13). Recently, several micro-sized HBOCs, the size of which is similar to human RBCs, have been developed using layer by layer assembly (14,15), a sacrificial template (16–21), conjugation to microparticles (22), and printing technology (23). We also have developed novel micro-sized HBOCs by cross-linking bovine Hb (bHb)- and bovine serum albumin (BSA)-encapsulating emulsions formed via Shirasu porous glass (SPG) membrane emulsification (24,25). This bHb–BSA microsphere shows good oxygen dissociation behavior, while preventing cellular uptake by the relatively large diameter, precisely controlled via the SPG membrane emulsification technique.

For advancing practical use of micro-sized HBOCs, one concern is the use of animal-derived materials. To the best of our knowledge, previously reported micro-sized HBOCs, including our HBOCs, used Hb or carrier material extracted from animals, such as bovine material (14–25). However, the use of animal-derived material inevitably has a potential risk for infectious disease, such as bovine spongiform encephalopathy. Because some regenerative medicine therapies are becoming realized in the clinical stage, more focus is now placed on the safety and quality of regenerated tissues (26–28). Along this line, animal-derived materials in micro-sized HBOCs need to be replaced with safer material for their use in 3D tissue culture *in vitro*. In this study, we developed human-derived, Hb–albumin microspheres using the SPG membrane emulsification technique. Human Hb (hHb) and albumin (HSA) were extracted from donated human blood and used for fabrication of microspheres. The potential of obtained hHb–HAS microspheres as oxygen carriers was investigated using a hypoxia-responsive cell line that we recently established, which expresses EGFP in response to hypoxic conditions (29).

MATERIALS AND METHODS

Materials Human red blood cell (hRBC) preparations from donated blood was provided by the Japanese Red Cross Society with approval by the ethics

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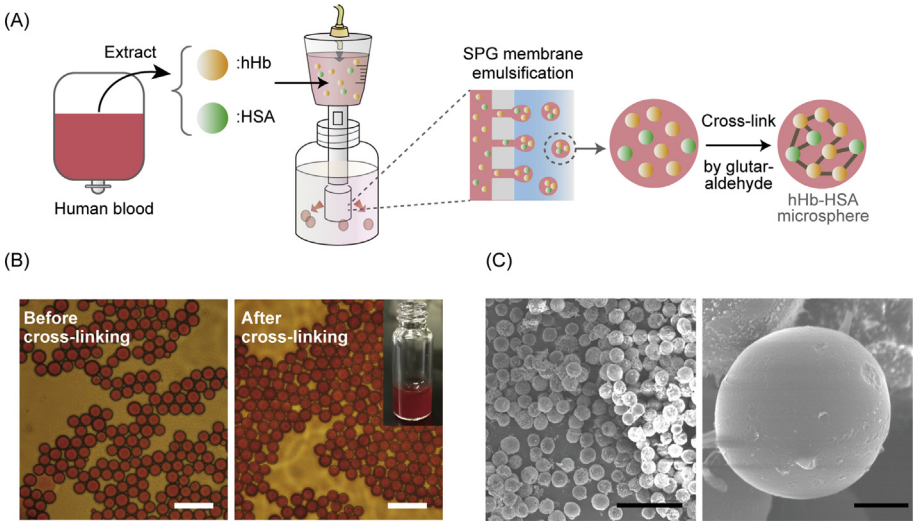

FIG. 1. (A) Schematic illustration of fabrication of hHb–HSA microspheres. The dispersed phase containing hHb and HSA was emulsified using an SPG membrane, followed by cross-linking with glutaraldehyde. (B) Optical microscopic images of hHb-HSA emulsions before and after cross-linking. Emulsions before crosslinking were dispersed in kerosene, while those after cross-linking (i.e., hHb–HSA microspheres) were dispersed in pure water. The inset shows the appearance of aqueous hHb–HSA microsphere solution. The scale bar is 50 μm. (C) SEM images of hHb–HSA microspheres. Left: low magnification image of various particles. The scale bar is 500 μm. Right: enlarged images of one representative particle. The scale bar is 3 μm.

committee of the Faculty of Medicine, The University of Tokyo (no. 10412) and the Ministry of Health, Labour and Welfare, Japan (27J0029). Human albumin preparation from donated blood was also provided by Japan Blood Products Organization with the above-mentioned approval. Tetraglycerol condensed ricinoleate (TGCR) was kindly provided by Sakamoto Yakuhin Kogyo Co. (Osaka, Japan). Toluene, kerosene, glutaraldehyde, glycine, BSA, and penicillin-streptomycin-amphotericin B were purchased from Wako Pure Chemical Industries (Osaka, Japan). Dulbecco's modified Eagle's medium (DMEM) was purchased from Sigma–Aldrich (St. Louis, MO, USA). Dialysis membrane (Spectra/Por, molecular weight cut off = 6000–8000 Da) was purchased from Spectrum Laboratories Inc. (Rancho Dominguez, CA, USA). Fetal bovine serum (FBS) was purchased from Thermo Fisher Scientific Inc. (Waltham, MA, USA). The Cell Counting Kit-8 was purchased from Dojindo (Kumamoto, Japan).

Extraction of hHb from hRBC preparations hHb was extracted from hRBC preparations based on our previously reported procedure for bHb extraction (24). First, 140 ml of pure water was added to 140 ml of hRBC preparation, and stirred for 20 min at 4°C to induce hemolysis. The solution was then transferred to a separating funnel to which 70 ml of toluene was added. The funnel was shaken for 5 min and then left to stand for 3 days at 4°C. After the separation, the water phase was taken out from the funnel, followed by ultracentrifugation at 20,000 rpm and 4°C for 1 h. Supernatant was dialyzed against pure water at 4°C for 1 week, and then preserved at −80°C until later use.

Preparation of hHb–HSA emulsions by SPG membrane emulsification W/O emulsions containing hHb and HSA were prepared using an SPG membrane with 5-μm pore size (SPG Technology Co. Ltd., Miyazaki, Japan). A total of 20 wt% of hHb and 10 wt% HSA were dissolved in 2 ml PBS as the dispersed phase, while 20 ml kerosene containing 1 wt% TGCR was used as the continuous phase. The dispersed phase was pressurized using nitrogen gas through the SPG membrane to the continuous phase under stirring at 150 rpm. This resulted in formation of W/O emulsions containing hHb and HSA. Then a volume of 20 μl of 25 w/v % glutaraldehyde was added to the emulsified solution and stirred for 1 h at room temperature. The reaction was quenched by addition of 0.1 M glycine, followed by washing with pure water. The obtained hHb–HSA emulsions and microspheres were observed by optical microscopy and scanning electron microscopy (SEM) (S-900; Hitachi, Tokyo, Japan). The size distribution of the emulsions and microspheres was measured by a laser diffraction particle size analyzer (LA-350; Horiba, Kyoto, Japan) and optical microscopic images. Size change of hHb–HSA microspheres during the incubation in DMEM with 10% FBS at 37°C was examined for 8 days using the laser diffraction particle size analyzer.

For comparison, 20 wt% bHb-10 wt% BSA microspheres were also fabricated with the same way as described above except for the use of bHb and BSA. Viscosity of the dispersed phase for both hHb–HSA and bHb-BSA microspheres was measured with a DV-II+Pro cone-type viscometer (Brookfield AMETEK, Inc., Middleboro, MA, USA).

Measurement of oxygen dissociation behavior The oxygen dissociation curve (ODC) of extracted hHb and hHb–HSA microspheres was measured by using a Hemox analyzer (TCS Scientific, New Hope, PA, USA). Sweeping speed of PO_2 was set at 15 min from 150 to 0 mmHg. Using the obtained ODC, two characteristic values were calculated: P_{50} and the Hill coefficient n. P_{50} was defined as the PO_2 when oxygen saturation reached 50% in the ODC, representing the binding affinity with O_2. The n value indicates the cooperative extent of the four subunits in the Hb molecule, and was calculated via the Hill equation. Oxygen dissociation and association curve of hHb–HSA microspheres during repeated oxygenation and deoxygenation was also measured using the Hemox analyzer.

Evaluation of methemoglobin formation by the cyanomethemoglobin assay Resistance of hHb–HSA microspheres against methemoglobin (MetHb) formation was evaluated by incubating 1 mg/ml suspension of the microspheres in PBS at 37°C. Extracted hHb was also used as a control. On days 0, 4, and 7 after starting incubation, the percentage of MetHb in the total hHb contained in the hHb–HSA microspheres was determined by the cyanomethemoglobin assay, according to previous reports (24,25,30,31).

Evaluation of the ability of oxygen supply from hHb–HSA microspheres using hypoxia-responsive HeLa cells The ability of oxygen supply from hHb-HSA microspheres was evaluated by genetically engineered HeLa cells that express EGFP in response to hypoxia (29). Hypoxia-responsive HeLa cells were grown in DMEM containing 10% FBS and 1% penicillin-streptomycin-amphotericin B. Cells were maintained in a humidified incubator at 37°C with 5% CO_2. For evaluation of oxygen supply, the cells were seeded on a 24-well glass-bottom microplate at a density of 2.0×10^4 cells per well and kept overnight at 37°C in 5% CO_2. The culture media were replaced with media containing different concentrations of hHb–HSA microspheres. Culture media containing an equivalent amount of extracted hHb was also used for comparison. The cells were then cultured under 19% or 2% O_2 in a gas-controlled CO_2 incubator (MCO-5M; Panasonic, Osaka, Japan) for 48 h. After incubation, hypoxia-induced fluorescence from the cells was observed using a fluorescent microscope (IX73; Olympus, Tokyo, Japan). The fluorescence was also measured using a fluorescent microplate reader (2030 ARVO V3; PerkinElmer, Waltham, MA, USA). The obtained fluorescence intensity was then normalized to the number of cells measured via WST assay using the Cell Counting Kit-8. The absorbance at 450 nm by formazans, produced by active mitochondria, was measured with the microplate reader. The relative number of cells in each well (N_R), normalized to the number of cells cultured without microspheres at 19% O_2 as a control, was calculated as the ratio of the absorbance value to that of the control. The fluorescent intensity I_{well} was divided by N_R to obtain the normalized fluorescence intensity I_{norm}.

RESULTS AND DISCUSSION

Fabrication of hHb-HSA microspheres hHb was extracted from donated hRBC. W/O emulsions containing 20 wt% hHb and 10 wt% HSA were prepared using an SPG membrane with 5-μm pore size (Fig. 1A). Fig. 1B shows optical microscopic images of the formed emulsions. Monodispersed emulsions with a spherical shape were observed. HSA and hHb encapsulated in the emulsions were then

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