博士論文　(要約)

Development of new artificial oxygen carriers for tissue engineering via SPG membrane emulsification technique

（ＳＰＧ膜乳化法を用いた組織工学用新規人工酸素運搬体の開発）

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化学システム工学専攻

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Chapter 1: General Introduction

Organ loss or organ failure is one of the severe threats to human life, as some of the damaged organs can not be repaired or regenerated. Recently, tissue engineering is expected to supply regenerated tissues for transplantation instead of organs from donors. By combining cells, scaffolds, and cell signaling, scientists have been able to engineer different types of tissues and even organs.

Sufficient oxygen supply is an important criteria for obtaining a significant mass amount of regenerative tissues. This is especially for highly metabolic cells, such as liver cells, as they are closely related to regulation of cell survival, differentiation, and function. However, oxygen is often the limiting nutrient for cells grown in vitro due to the sparingly soluble amount in culture medium. One strategy to increase oxygen solubility in culture medium without raising oxygen tension is to apply artificial oxygen carriers.

There are currently three types of artificial oxygen carriers: hemoglobin-based oxygen carriers (HBOCs), perfluorocarbons (PFCs) and silicone-based oxygen carriers. They are different in various aspects, such as occurrence, chemical structure, oxygen association/dissociation profiles, oxygen capacity, physical and chemical properties. Application of artificial oxygen carriers to culture system to increase oxygen solubility is expected.

In order to obtain a successful cultivation, oxygen carriers of appropriate sizes should be considered, since cytotoxicity might occur due to the uptake of nano-sized HBOCs by cells, and micro-sized oxygen carriers are thought to avoid cellular uptake. It is also a problem if oxygen carriers have a broad size distribution, including obstruction of fluid channel that limits the oxygen delivery, and oxygen capacity will be difficult to estimate. Therefore, methods which can precisely control the size and size distribution of oxygen carriers are needed.

Shirasu porous glass (SPG) membrane emulsification is a promising method to prepare uniform-sized emulsions, and subsequent microspheres, with the merits of scale up potential and low cost as compared to other particle size-control technique. Many kinds of microspheres with the coefficient of variation (CV) around 10% for different purposes have already been fabricated. Artificial oxygen carriers made by SPG membrane emulsification technique is thought to solve problems of size control and size distribution.

The objective of this thesis is to develop uniform micro-sized oxygen carriers by SPG membrane emulsification technique for tissue engineering applications. Two types of oxygen carriers will be developed in this thesis: HBOCs and silicone-based oxygen carriers, due to the high oxygen capacity and stability of each material, respectively. These oxygen carriers will be applied to the oxygenation of cells, and the efficacy will be evaluated in terms of cell viability, metabolism, and function.

Chapter 2: Development of hemoglobin-based oxygen carriers (HBOCs) and their properties

A new type of HBOCs composed of extracted bovine hemoglobin (bHb) and bovine serum albumin (BSA) was developed using SPG membrane emulsification technique. The disperse phase containing 10 wt% bHb and 5–20 wt% BSA was pressurized by nitrogen gas through the SPG

membrane to the continuous phase that contained kerosene and tetraglycerol condensed ricinoleate (TGCR). Incorporation of BSA improved the stability of the emulsions. The resultant emulsion droplets were subsequently cross-linked by glutaraldehyde to form microspheres, i.e., bHb_{10}-BSA_m microspheres (m = 5, 10, 15 and 20 as per the composition of disperse phase). The average diameters (D_{avg}) of bHb_{10}–BSA_m microspheres were successfully controlled at around 5 μm with a CV of around 10%. In addition, bHb and BSA were successfully assembled into microspheres while maintained their activity according to FT-IR and UV-Vis spectrum. These microspheres show the ability to bind and release oxygen, with P_{50} values ranged from 8.08 to 11.60 mmHg, and their activities can be maintained by preserving at -80 ℃, thus exhibiting great potential as oxygen carriers in tissue engineering applications.

Chapter 3: Size-control of HBOCs and their effects on cellular uptake and cell viability

In this chapter, uniform bHb_{10}-BSA_{10} microspheres with D_{avg} ranging from 1.2 to 18.3 μm were fabricated using the SPG membrane emulsification technique mentioned in chapter 2. These microspheres were then exposed to RAW264.7 (mouse leukemic monocyte macrophage cell line), HepG2 (human hepatocellular carcinoma cells) and HUVEC (human umbilical vein endothelial cells) to investigate the interaction between different sizes of HBOCs and cells in terms of cellular uptake and viability. According to fluorescence-activated cell sorting (FACS) results, cellular uptake of the bHb_{10}-BSA_{10} microspheres by RAW264.7 was observed at a diameter below 5 μm. However, uptake of the microspheres by HepG2 and HUVEC were not observed at any diameter, coinciding with the reactive oxygen species (ROS) assay results. The size dependency of bHb_{10}-BSA_{10} microspheres on biocompatibility was observed, where cytotoxicity decreased with increasing microsphere diameter in all three cell lines. bHb_{10}-BSA_{10} microspheres of 18.3 μm showed good cellular compatibility regardless of the oxyHb percentage. The speculation of this size dependency on cell viability is as follows: bHb_{10}-BSA_{10} microspheres and cells were under static culture conditions during the MTT assay, thus it is likely that the microspheres underwent sedimentation onto the cell surface, resulting in direct contact with the cells. Due to the high surface-to-volume ratio for small microspheres with diameters between 1.2 and 4.9 μm, more Hb-mediated ROS were transferred from the microspheres to the cells and caused cytotoxicity. However, large microspheres with diameters of 9.8 and 18.3 μm might not have come into direct contact with the cells to the same extent as the small microspheres.

The time to release all the oxygen from microspheres depends on their size. This was verified by changing the measuring time of oxygen dissociation curves (ODCs). Based on the Thiele modulus, it is shown that the rate-limiting step switch from surface reaction to internal diffusion for microspheres with diameter larger than 10 μm.

Chapter 4: Development of poly-TRIS-g-MPC oxygen carriers

A silicone-based oxygen carriers was developed in this chapter. Methacryloxypropyl tris(trimethylsiloxy)silane (TRIS), a silicone monomer with a high content of trimethylsiloxy groups, can enlarge the intersegmental distances in the resulting polymers and enhance oxygen solubility,

which is a promising candidate for making oxygen carriers. However, poly-TRIS microspheres show aggregation in aqueous solution due to its hydrophobic surface, and this hydrophobic surface-induced shear stress might damage cells. Therefore, it is necessary to render the surface hydrophilic. 2-methacryloyloxyethyl phosphorylcholine (MPC), a zwitterionic biomimetic monomer, should be useful for surface modification as it forms polymer that possess biocompatible and low friction properties.

The disperse phase containing TRIS monomer and photo-initiator was pressurized by nitrogen gas through the SPG membrane to the continuous phase containing sodium dodecyl sulfate (SDS) and water to form TRIS emulsions. An anionic surfactant is preferred to a nonionic surfactant for TRIS emulsions because of the silanol groups on the SPG membrane surface. The optimum concentration of SDS is at least 1 wt% in the continuous phase. The emulsions were subsequently undergoing UV-initiated polymerization to form the poly-TRIS microspheres. The FT-IR spectrum indicated that poly-TRIS microspheres were successfully synthesized, with CV around 12% by SEM micrographs. By varying the SPG membrane pore size, the average diameter of the resulting TRIS emulsion droplets and poly-TRIS microspheres were 2.47 and 2.30-fold the membrane pore size, respectively.

Benzophenone (BP) adsorbed poly-TRIS microspheres were suspended in MPC monomer solution, after polymerization by irradiating of UV lamp, poly-MPC was grafted to the poly-TRIS microspheres to form the poly-TRIS-g-MPC microspheres, as confirmed by FT-IR spectrum. In addition to showing dispersity in water, the oxygen capacity of the poly-TRIS-g-MPC microspheres was also determined.

Chapter 5: Evaluation of oxygen carriers in cell culture

In this chapter, bHb_{10}-BSA_{10} microspheres and poly-TRIS-g-MPC microspheres were applied to the oxygenation of HepG2 cells in 20% and 1% oxygen through shaking culture. The oxygenation efficiency of the carriers was evaluated in terms of cell viability, metabolism, and function.

To reveal the actual performance of oxygen carriers, some improvements are needed. The oxygenation efficacy might be overestimated due to the active metabolism of HepG2 cells, especially in hypoxia. The 3-bromopyruvate (3-BP) is a pyruvate analog which functions as a glycolytic inhibitor that affects cancer cells by disrupting their energy metabolism; the efficiency of the carriers using 3-BP to eliminate active glycolysis effect was examined. In order to avoid the cytotoxicity induced by direct interaction between bHb_{10}-BSA_{10} microspheres and cells, Transwell was also applied.

The bHb_{10}-BSA_{10} microspheres showed toxicity rather than efficacy to cells while exposed to cells in 20% oxygen without the application of Transwell. However, efficacy was shown with the application of Transwell and 3-BP in 1% oxygen. The efficacy of poly-TRIS-g-MPC microspheres was similar as compared to control in 20% or 1% oxygen.

Numerical simulations were performed to understand the oxygen transport: 20 mg/mL of bHb_{10}-BSA_{10} microspheres would enhance oxygen flux by 1.5-fold and 27-fold in 20% and 1% oxygen, respectively. The oxygen capacity of poly-TRIS-g-MPC microspheres can be estimated by

Henry’s law. Subsequently, the enhancement of oxygen flux using 10 mg/mL of poly-TRIS-g-MPC microspheres was 1.3-fold and 1-fold in 20% and 1% oxygen, respectively.

Chapter 6: General conclusions and future perspectives

The results obtained in each chapter are summarized, and future perspectives of the present study are also proposed.