Synthesis of Hemoglobin-Based O_2 Carriers and Albumin-Based Plasma Expander as Blood Substitutes
血液代替物としてのヘモグロビンを用いた人工酸素運搬体とアルブミンを用いた人工血漿増量剤の合成

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This thesis describes the synthesis, structures, functions, safety, and efficacy of three artificial O_2 carriers and one artificial plasma expander. In Chapter 1, the general introduction on current status and issues of transfusion therapy were described. Japan's blood shortages will reach approximately 650,000 people per year in 2025 because of the declining blood donor population caused by the low birthrate and aging population, as estimated by Japan's Ministry of Health, Labour and Welfare. Disasters might also engender blood shortages. The shortage of red blood cell (RBC) products is particularly related to the short preservation limit RBC products. Consequently, RBC substitutes that can be stored for a long period must be developed. Komatsu *et al.* have previously synthesized a core–shell protein cluster comprising hemoglobin (Hb) in the center and three human serum albumins (HSA) at the periphery, a hemoglobin-albumin cluster (Hb-HSA_3) as an RBC alternative. Its safety was assessed using 20% exchange transfusion experiments using rats.

Chapter 2 described the efficacy of Hb-HSA_3. Administration of Hb-HSA_3 solution in anesthetized rats in hemorrhagic shock recovered circulatory parameter and blood gas parameter to similar levels of rats in the shed autologous blood group. All results indicate that the artificial O_2 carriers exert a resuscitative effect which is equivalent to that of whole blood. Serum biochemical tests revealed no acute toxicity and/or negative side effect of Hb-HSA_3. Actually, AST and ALT tended to increase by 6 hr after administration, but they returned to their initial concentrations within 7 days. The influence on liver function is temporary. This study has demonstrated that the Hb-HSA_3 solution can be attractive RBC alternative for resuscitation from hemorrhagic shock and for treatment of acute blood loss.

One issue related to Hb-based O_2 carriers is the decrease in the O_2 transport capacity by autoxidation of Hb. To overcome this shortcoming, particles made of stroma-free hemoglobin containing natural antioxidant enzyme catalase (SFHbNP) were synthesized in chapter 3. The scanning electron microscopy, transmission electron microscopy, and dynamic light scattering measurements revealed the formation of uniform particles with approximately 100 nm diameter. SFHbNP exhibited high O_2 affinity (P_{50} = 8 Torr) compared to the value of RBC. Even in H_2O_2

solution, SFHbNP formed a very stable O_2 complex, thereby demonstrating its high antioxidant ability. The SFHbNP showed good compatibility with blood components, RBCs, platelets, granulocytes, and monocytes revealed by in vitro inspections. The SFHbNP formulation having antioxidant ability can be preclinically tested further as an artificial O_2 carrier.

The shortage of blood available for veterinary medicine is actually more severe than human blood shortages. Japan, as a pet superpower, faces annually increasing demand for veterinary medicine, but no blood banks exist for animal use. Consequently, poly(2-ethyl-2-oxazoline)-conjugated Hb (POx-Hb) was synthesized as an artificial O_2 carrier for animals in chapter 4. POx is a biocompatible water-soluble polymer. The average number of POx bonds was 6, and POx conjugation did not affect the secondary structure of Hb. The POx-Hb solution shows high O_2 affinity (P_{50} = 9 Torr). Anesthetized rats in hemorrhagic shock were resuscitated compeletely by administration of POx-Hb solution. The circulatory parameters were recovery to similar levels of the shed autologous blood group. Serum biochemical tests revealed no acute toxicity and or negative side effect of POx-Hb. Organ morphology changes observed 2 hr after resuscitation were recovered after 7 days, indicating that organ damage was temporary. This study has demonstrated that the POx-Hb solutions can be attractive alternatives to RBC for resuscitation from hemorrhagic shock.

Because the supply of albumin products for dogs and cats is insufficient, veterinarians need not only RBC substitutes, but also plasma substitutes. Therefore, POx-conjugated porcine serum albumin (POx-PSA) was also synthesized as an artificial plasma expander for animals in chapter 5. The POx-PSA solution ([PSA] = 5 g/dL) showed moderately high COP (36 mmHg), thereby it is suitable for securing circulating blood volume. The POx coating of PSA increased circulation lifetime in rat for 2 times relative to the naked PSA. As expected, anti-PSA IgG antibody was not generated after administration of POx-PSA solution into rats. It was revealed an excellent immunological stealth capability of POx wrapping. It is particularly interesting that no anti-POx IgG was produced either. This result was in sharp contrast to the fact that the injection of PEG-PSA induced the production of anti-PEG IgG. When the POx-PSA solution was administered intravenously in dogs, no signs of serum biochemical or hematological alterations, or overt deterioration in animal health were observed. Overall, POx-PSA possesses three important beneficial features (i) low immunogenicity, (ii) long-term circulation, and (iii) adequate in vivo safety for clinical use. The POx-PSA solution can be of great medical importance as potential artificial plasma expander in diverse veterinary medicine situations.

Chapter 6 described conclusions and future prospects of this thesis. All four blood substitutes synthesized in this study present important potential for practical use.