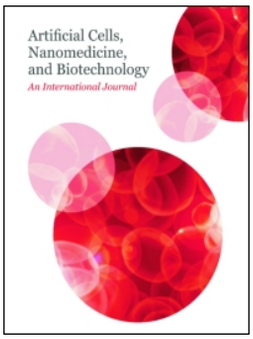

Artificial Cells, Blood Substitutes, and Biotechnology

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Poster Presentations

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ARTIFICIAL CELLS, BLOOD SUBSTITUTES, AND BIOTECHNOLOGY
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Poster Presentations

Properties of Perfluorochemical Emulsions in the Bloodstream

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The PFC dispersions are known to absorb lipids while circulating in the bloodstream. The composition of absorbed lipids depends on the chemical structure of PFC forming the nucleus. Each compound and their mixtures have affinity to certain classes of lipids and acyl radicals. PFD prefers middle chain acyls, PMCP – long chain acyl groups. Thus PFC dispersions can interfere into the lipid transport process in the bloodstream. Among lipids absorbed by PFD/PFTPA dispersions the amount of phosphatidylcholine consists of 66% (of total phospholipids (PL) and palmitic acid – 44% (of total fatty acids). The infusion of PFD/PFTPA emulsion with YPL as an emulgator to rats causes the reduction of PL synthesis in lungs. On the contrary the absence of YPL in PFC dispersions results as an intensification both of lipid absorption and PL synthesis in lungs. So lungs serve as a source of plasma PL and PFC infusion may influence the lung tissue. These results are in accordance with findings of lung destruction in neonates after massive infusion of fat emulsions.

Effects of Perfluorocarbon Blood Substitute ''Perftoran'' on Treatment of Acute Forms of Virus Hepatitis B

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Under treatment and control there were 157 patients with acute forms of virus hepatitis B. Out of them 79 patients were treated with perfluorocarbon

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blood substitute ''Perftoran'' in the course of complex intense therapy. The preparation was administered intravenously by dropping in a dose of 400–800 ml/day over a period of 2–6 days. A clear clinical effect was observed, which was characterized by a significantly quicker clinical recovery and normalization of bilirubin content and ALT activity in the group of patients treated with pertoran. The duration of patients' stay in the intense therapy ward was reduced by more than twofold.

Antioxidant, immunomodulating and antiinflammatory effects of Perftoran were also registered during the treatment of acute forms of virus hepatitis B. These effects were expressed in decreasing myeloperoxidase prooxidant activity of neutrophil granulocytes and induction of antioxidant protection factors: reduced glutathione, catalase and glucose-6-phosphatedehydrogenase in blood erythrocytes, normalization of immunological factors and proinflammatory cytokines content in blood serum (IL-1, IL-6, IL-8, TNF-α).

The conclusion was made, that it would be expedient to include the perfluorocarbon emulsion ''Perftoran'' in the course of the intense therapy while treating the acute forms of virus hepatitis B.

Effect of Perfluorocarbon (PFC) Blood Substitutes on Pulmonary Hyperinflation in Rabbits

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Oxycyte is a new PFC-based blood substitute and therapeutic oxygen carrier that has been formulated with a PFC selected to make a stable, sub-micron emulsion without having to add a second, less desirable PFC, and to avoid the pulmonary toxicity and environmental ozone depletion risks associated with other PFC's. The present study was done to assess the risk of pulmonary toxicity, specifically hyperinflated, noncollapsible (HFNC) lungs, with Oxycyte. Male New Zealand White rabbits weighing 2.9 to 3.1 kg were given an intravenous infusion of 10 ml/kg of a 60% w/v Oxycyte emulsion, or 10 ml/kg of a 60% perflubron emulsion. Control animals received an equal dose of normal saline. All animals were sacrificed on day 5. Their lungs were immediately removed, the trachea's tied off , and weighed. Fluid volume displacement was then determined by completely submerging lungs tied to a sinker in a beaker of saline filled to a pre- marked level. Relative lung volumes in ml/kg of body weight were 9.66 (SD 2.46) in Oxycyte treated animals, 21.15 (SD 1.49) in perflubron treated animals, and 10.63 (SD 5.69) in saline control animals. HFNC lungs were pale in color, and failed to collapse when the chest cage was opened. Perfluorodecalin has been shown in previous studies to cause HFNC.

Preliminary laboratory and animal studies suggest that Oxycyte is stabile, safe, and effective in carrying and off-loading oxygen. We conclude that Oxycyte warrants further study as an improved blood substitute and therapeutic oxygen carrier.

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Do Perfluorocarbon (PFC) Emulsions Modify Red Blood Cell Behavior in a Hemodiluted Rabbit?

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We report on the effect of a newly-formulated, small-sized ($\sim$ 100 nm) and highly stable PFC emulsion on the in vitro and in vivo rheological behavior of red blood cells, as compared to the effect of plasma and Gélofusine®. For each shear rate investigated, the in vitro viscosity of blood samples (BS) diluted with the 60% w/v concentrated PFC emulsion (Hct 0.20 L/L) was decreased as compared to whole blood (WB, Hct 0.40 L/L), but slightly less than that of BS diluted with the controls (Hct 0.20 L/L). For both the emulsion and controls, in vitro rouleaux formation showed a decrease of the primary aggregation time and of minimal shear rate that induces partial and total disaggregation, as compared to WB. S_{10} (relative area above the curve during the first 10 sec) was stable and the final aggregation time was similar for the emulsion and controls. These results were therefore attributed to the decrease of Hct. In the in vivo experiments, BS were withdrawn 5 min, 1, 2 and 3 hours after resuscitation by the PFC emulsion (16.2 g PFC per kg body weight) of anaesthetized rabbits that had undergone an acute hemorrhagic shock. The in vivo viscosity decreased, but for the first BS, and then remained stable. The aggregation parameters were similar to those of the in vitro study, but for S_{10} for which a more pronounced decrease was observed with the PFC emulsion than with controls, expressing a hypoaggregated state. We conclude that this new PFC emulsion does not induce any fundamental rheological perturbation, even when a large dose is administrated (about *six times* the intended clinical dose).

Improved Tissue Oxygenation Capacity of a New Generation Perfluorocarbon Emulsion After Acute Hemorrhagic Shock in Rabbits

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The ability to deliver oxygen to tissues of a newly-formulated, small sized ($\approx$ 100 nm) and highly stable perfluorocarbon (PFC) emulsion formulated with a semifluorinated alkane was evaluated. In an anaesthetized and ventilated (FiO_2 = 1.0) rabbit model of resuscitation from acute hemorrhagic shock (50% of blood), we compared the effects of the new generation PFC emulsion, diluted in a 33% albumin (50 g/L in Ringer) solution (16.2 g PFC per kg body weight), to Gelofusine® (a modified fluid gelatine), on hemodynamic parameters, blood gases and skeletal muscle O_2 pressure ($PtiO_2$). No significant differences between both reperfusion

solutions were observed on hemodynamic parameters and on blood gases. The $PtiO_2$ increased rapidly when reperfusion was done with the PFC emulsion, exceeded base line values (59 vs 34 mmHg) and remained high during the three hours of observation, while with Gelofusine the $PtiO_2$ did not return to the basal value (38 vs 24 mmHg) and remained relatively stable. The interstitial cellular concentrations and blood concentrations of lactate and pyruvate (anoxic cells metabolites) were also measured in both case of the PFC emulsion and control solution. So, this new PFC emulsion does not induce perturbations on the hemodynamic parameters and allows a better tissue oxygenation after an hemorrhagic shock.

O_2-Coordination and Biocompatibility of Albumin-Heme as a Synthetic O_2-Carrier

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Recombinant human serum albumin (rHSA) incorporating tetraphenylporphyrinatoiron derivative bearing an imidazolyl group (albumin-heme, rHSA-FeP) is an synthetic hemoprotein, which can bind and release O_2 reversibly under physiological conditions (Tsuchida et al., 1999). We report herein the O_2-coordination structure and compatibility of rHSA-FeP with blood.

The infrared spectroscopy showed the vibration stretching mode of the O_2 coordinated to rHSA-FeP at 1158 cm^{-1}, which is identical to those observed in other synthetic hemes. Magnetic circular dichloism spectrum of rHSA-FeP also exhibited the formation of O_2-coordinate ferrous low-spin complex. We concluded an end-on type coordination of O_2 to rHSA-FeP.

After mixing rHSA-FeP ([rHSA]: 5 wt%) solution with whole blood, it has been found no change in the numbers of white blood cells and red blood cells for 6 hrs at 37°C. We are certain that rHSA-FeP has good compatibility in vitro, and satisfies the initial clinical requirements for red blood substitute.

Tsuchida, E., Komatsu, T., Matsukawa, Y., Hamamatsu, K., Wu, J. (1999). *Bioconjug. Chem.* 10:797–802.

Serum Albumin Included Iron-Porphyrins as a Synthetic O_2-Carrying Hemoprotein

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The serum albumin has been used as a plasma expander and a therapeutic drug carrier in the circulatory system. From a viewpoint of clinical application, it is of great interest to develop an O_2-carrying albumin, which could be of extremely medical importance as a new class of artificial red blood cell. However, it has been established that the native albumin-protoheme complex is inactive as an O_2 transporter.

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We have found that tetraphenylporphinatoiron(II) derivative with a covalently linked proximal base [Ppiv(Im)] is included into recombinant human serum albumin (rHSA), providing synthetic hemoprotein [rHSA-Ppiv(Im)] which can bind and release O_2 reversibly under physiological conditions (in aqueous media, pH 7.3, 37°C) like hemoglobin and myoglobin (Komatsu and Tsuchida, 1999). A series of similar porphyrins, involving protoporphinatoiron(II) derivatives with a covalently attached axial base, have been systematically incorporated into rHSA, and the O_2-coordination behavior was elucidated in terms of their porphyrin structures. In particular, we found that the histidine coordination gives long lifetime of the O_2-adduct complex.

Komatsu, T., Tsuchida, E. (1999). *Bioconjug. Chem.* 10:799–803.

A Recombinant Polymeric Hemoglobin as Artificial Oxygen Transporter

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We have used recombinant technology for obtaining physiologically competent polymers of tetrameric hemoglobin (Hb). Polymerization hinders extravasation through the endothelium, and the associated vasoactivity. The polymerization strategy is based upon S-S bonding of Cys residues introduced on the surface of the Hb molecules. We have constructed Hb Minotaur (α_A-β_{Bv}) made by human α-chains and bovine β-chains in which we have replaced Cys present at position α104 in HbA and β93 in HbBv, and introduced a Cys at position β9. This mutant, Hb Minotaur-P (α_AC104S-β_{Bv}C93A + A9C), forms a homogeneous polymer of 6–8 tetrameric molecules with a MW ~ 500kDa. It is resistant to denaturation, has increased heme affinity, and a P_{50} = 16 torr at 37°C at pH 7.4. In mice, the retention time of natural HbA it is of 0.5 h, for Hb Minotaur-P is increased to 3.0 h. Exchange transfusion with albumin and Hb Minotaur-P indicates that the infusion of the polymer is not associated with increased blood pressure or changes in the body temperature. After 24 hours the mice appeared to have normal behavioral activity. Although plasma is rich in reducing agents, no indication of de-polymerization is evident 1h after polymer injection into circulation. This polymer contributes to development of recombinant Hbs with therapeutic applications.

Modified Porcine Haemoglobin Hyperpolymers as an Oxygen Carrying Blood Additive

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The enormous clinical need for combating oxygen deficiencies without lack of blood, caused e. g. by an anaemia, local ischaemias and their complications like

stroke or myocardial infarction, or for use in pre-treatment tumour oxygenation, should preferably be met by an infusible hypo-oncotic artificial oxygen carrier (a 'blood additive').

We are developing an hypo-oncotic oxygen carrying blood additive made of pegylated porcine haemoglobin, highly cross-linked to (hyper-) polymers with a narrow distribution of molecular weights around 700,000 g/mol, obtained with a technical simple procedure in good yield.

The oxygen pressure at half saturation of these hyperpolymers is about 15 Torr, and Hill's index 2.0. They are fully compatible with human blood plasma, and at the intended therapeutical concentrations up to 30 g/L, their impact on oncotic pressure is low, and well tolerable regarding blood viscosity.

Preclinical pharmacological and toxicological studies have begun, where safety is evaluated and first results regarding the radiation sensitisation of solid tumours in animals are investigated. Furthermore, repeated applications to human volunteers were very well tolerated, no increase of transaminases (GOT and GPT), and no signs of an immunoreaction were seen.

Total Exchange Transfusion in the Rat with PEG-Conjugated Hemoglobin, Peg-Conjugated Albumin, or Pentastarch

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We have prepared a novel blood substitute with high O_2 affinity (~ 6 torr) by surface conjugation of maleimide-activated polyethylene glycol to human hemoglobin (MalPEG-Hb). Rats were continuously exchange transfused for 60 min, and monitored for 130 min or until death. Three test solutions were evaluated (n = 5 in each group): 1) MalPEG-Hb, 2) MalPEG-human serum albumin (MalPEG-HSA) made using the same PEG conjugation chemistry to match the physical properties of MalPEG-Hb but without the O_2 carrying capacity, and 3) pentastarch (PS) as a volume expanding control in the absence of PEG. None of the MalPEG-HSA or PS animals survived total exchange, and all were dead by 80 min (Hct $\sim 10-12$ %). In contrast, all animals that received MalPEG-Hb survived the entire exchange and observation period at undetectable Hct ($< 2\%$), plasma Hb ~ 3 g/dl, and total Hb ~ 3.5 g/dl. The animals' survival was predicted by their acid-base status. In the MalPEG-Hb group, pH remained constant, and lactic acid rose only when the Hct became undetectable. In MalPEG-HSA and PS groups, pH fell and lactic acid rose sharply at Hct $\sim 10\%$. Base excess showed a similar pattern; MalPEG-HSA and PS animals fell into negative values at Hct $\sim 10-12\%$, while MalPEG-Hb animals kept positive values until they reached Hct $< 2\%$. In conclusion, MalPEG-Hb is able to sustain life in the virtual absence of red blood cells. These results indicate that the beneficial properties of MalPEG-Hb cannot be due solely to PEG modification or volume expansion. In spite of its low P50, MalPEG-Hb is able to transport O_2 to tissue even at very low plasma and total hemoglobin concentrations.

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Perceptions of the Risk of Blood Transfusion and Donation in the UK: Sources of Reliable Information in Stakeholder Groups

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Clearer understanding of sources of accurate information on the benefits and risks of blood transfusion and donation will underpin improved strategies for targeting relevant messages to stakeholder groups. There is a need to determine what information sources stakeholders recognize as most useful and trustworthy. This study has identified sources of information on blood donation and transfusion (including blood substitutes) used by UK adult blood donors, anaesthetists, general practitioners (GPs) and healthcare journalists of both genders. A questionnaire survey was conducted in March–July 2000 involving (1) blood donors ($n = 250$), (2) GPs ($n = 88$), (3) anaesthetists ($n = 143$), (4) and journalists ($n = 20$). The mean ($\pm$ s.d.) age of respondents was 35.8 ± 12.6 years. Respondents scored, on a scale from 1 (of no use) to 7 (extremely useful), the value of information from principal sources (e.g. media, Web) in terms of providing useful and trustworthy information on blood use. Overall, medical sources, including information from blood donor services, were rated highest by all groups. Factor analysis of scores for each source of information loaded on 2 factors namely, popular sources and scientific sources (SS). GPs rated SS significantly ($P < 0.05$) higher than journalists, whilst anaesthetists and journalists rated SS lower ($P < 0.05$) than blood donors. These findings suggest that, in communicating risk information associated with blood use, utility and trust may be maximized using different communication strategies targeted to specific groups.

Adhesive Properties of rGPIbα Conjugated-Albumin Polymers and Phospholipid Vesicles on vWf-Immobilized Surface

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Albumin polymers (polyAlb) (Takeoka et al., 2000, 2001) and phospholipid vesicles bearing recognition proteins of platelet membrane have been evaluated as candidates for platelet substitutes. We conjugated recombinant glycoprotein; rGPIbα, which recognizes von Willebrand factor (vWf) to those carriers and studied in detail the differences between polyAlb and vesicles with the same particle size and the number, and the same amount of rGPIbα.

Under flow conditions, rGPIbα-polyAlb attached to the surface of the vWf-immobilized plate and then stopped. rGPIbα-latex beads and OsO_4-treated platelets also showed the similar adhesion to the polyAlb. On the other hand, rGPIbα-vesicles rolled

on the vWf surface in the direction of flow, like platelets. The number of rGPIbα-vesicles rolling on the vWf surface was constant, suggesting that the attachment rate and the detachment rate of the vesicle should be the same. The rolling velocity of rGPIbα-vesicles increased in accordance with the decrease of the membrane fluidity. The rolling phenomena of the rGPIbα-particles under flow conditions would be attributed to the fluidity of the particle surface where rGPIbα was conjugated.

Takeoka, et al., (2000). *Biomacromolecules* 1:290–295.
Takeoka, et al., (2001). *Biomacromolecules* 2:1192–1197.

Circulation Persistence and Biodistribution of the Hemoglobin-Vesicles (HbV) in Rats

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The phospholipid vesicles encapsulated hemoglobin solution (40 g/dL) (HbV) are useful materials as a red blood cells (RBC) substitute. We report on the circulation persistence and biodistribution of the HbV labeled with 99m-technetium (^{99m}Tc). The ^{99m}Tc-HbV was injected into rats from tail veins at 15% and 25% to blood volume. The circulation half-life ($t_{1/2}$) of ^{99m}Tc-HbV was determined to be 15 hrs and 24 hrs for 15% and 25% groups, respectively. While, the empty vesicles (control) was eliminated significantly faster ($t_{1/2}$ = 6 hrs) from blood circulation than HbV. The biodistribution data showed the major organs to eliminate the ^{99m}Tc-HbV and control vesicles from the blood circulation were liver, bone marrow, and spleen. The longer circulation life with increasing the injection dose was caused by decreasing the distribution ratio to liver. While, the remarkable difference of the circulation life between control vesicles and HbV was caused by the distribution into spleen. The distribution ratio of the control vesicles showed four times higher values than that of HbV. The large injection dose and encapsulation of Hb prolonged the circulation life of the HbV.

Characteristics of Bovine Hb as a Potential Source for Hb-Vesicles (HbV) as O_2 Carriers for Veterinary Use

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HbV is an artificial O_2 carrier in which a purified Hb solution is encapsulated with a lipid bilayer membrane. In this study bovine Hb (BHb) was tested as a source of HbV instead of human Hb (HHb), and compared the preparation process and characteristics

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of the BHbV and HHbV. The purification of BHb was effectively performed simply with an ultrafiltration system including a process for removing virus and scrapie agent. The removal ratio of the phospholipid components of bovine RBC was >99.99%, and the protein purity was >99.9%. The deoxy and carbonyl BHbs showed denaturation transition at 83 and 87C, respectively, which are higher than those of HHb (80 and 78C, respectively), and resistant to pasteurization (60C, 10 hrs). The purified BHb was concentrated to over 40 g/dL, and encapsulated with a phospholipid bilayer membrane to form BHbV with diameter of about 280 nm. Its O_2 affinity (P_{50}) was regulated by coencapsulation of appropriate amount of Cl^- which binds to BHb as an allosteric effector, in the range 16–28 Torr, being comparable with human RBC (P_{50} = 28 Torr). This is quite simple in comparison with HHb which requires phosphate derivatives such as PLP as a substitution of 2,3-DPG. The viscosity and COP of the BHbV when suspended in 5% albumin are 3.5 cP and 20 Torr, respectively, which are comparable with those of human blood. In conclusion, BHb can be used as a source for HbV, because of not only its abundance in cattle industries but also its advantages of purification process, thermal stability, and regulation of O_2 affinity in comparison with HHb.

Influence of Hb-Vesicles (Artificial O_2 Carriers) on Serum Clinical Chemistry

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Hb-vesicle (HbV, diameter = 251 ± 80 nm) is an artificial O_2 carrier for the substitution of the function of RBC. We clarified the interference of the HbV on serum clinical chemistry, and established a pretreatment method to avoid such an interference.

The HbV or Hb solution was mixed with a pooled human serum and the magnitude of their interference effect on a total of 30 analytes was studied. The mixture of the HbV and serum was ultracentrifuged (50,000g, 20 min) to remove the HbV particles as a precipitate and the same analytes in the supernatant were measured. The removal of HbV was also performed by centrifugation (2,700g, 30 min) in the presence of dextran (Mw. 200 kDa).

HbV showed interference for most of the analytes which was similar or more serious in comparison with the Hb solution. This is due to the light absorption of Hb in HbV and the light scattering from the suspension, and the components of HbV participate in the chemical reaction of the assays. On the other hand, the pretreatments to remove the HbV diminished the interference for most of the analytes. This will be one advantage of HbV in comparison with acellular chemically-modified Hb solutions.

Growth and Antioxidant Status in Cells Cultured with Bovine Haemoglobin Solution

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Supplementation of medium with gas-carrying perfluorocarbon liquids or haemoglobin (Hb) solutions, enhances biomass of cultured cells, but little attention has focused on the responses of cellular oxygen-detoxifying systems. Changes in reactive oxygen-scavenging enzymes were assessed in cotton cells (*Gossypium herbaceum* cv. Dhumad) following culture in media with bovine Hb solution (*Erythrogen*™; Biorelease, USA) at 1: 100–1: 1000 (v:v). After 25 d of culture, mean (± s.e.m.) fresh and dry weights (f/d.wt.) of cells were significantly ($P < 0.05$) greater in medium with 1: 750 and 1: 1000 (v:v) *Erythrogen*™, compared to controls. Thus, with 1: 750 (v:v) *Erythrogen*™, mean cell f/d.wt. were increased by 45 and 31%, respectively. Total soluble cellular protein increased by 141%, 176% and 191% with *Erythrogen*™ at 1: 50, 1: 750 and 1: 1000 (v:v), respectively. Catalase and glutathione reductase decreased significantly ($P < 0.05$) following the addition of low concentrations (1: 1000 : 1: 750 v:v) of *Erythrogen*™ to medium. Increasing *Erythrogen*™ to 1: 100 (v:v) caused a concomitant increase in catalase to a maximum of 62% over control. Mean total superoxide dismutase activity increased linearly with increasing *Erythrogen*™ concentration, reaching a maximum mean value of over 2-fold greater than control with *Erythrogen*™ at 1: 100 (v.v). A similar trend was observed for cellular H_2O_2 content, which was almost double that of control with 1: 250 (v:v) *Erythrogen*™. Thus, culture of cotton cells with Hb solution causes significant changes in cellular oxygenation status sufficient to modify antioxidant status.

Study on Condition for Cross-Linking of HB by Glutaraldehyde

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In the development of Hb-based red cell substitute, cross-linking of Hb is an important procedure. On this procedure many reports and patents have been issued. In our experiments with cross-linking of Hb, there occurred such problems as low polymeric rate, wide distribution of molecule weight (M.W.) of the reaction product, and production of ultra-high-M.W. polymers. To deal with these, we carried out an orthogonal test on condition factors which included PH value, Hb concentration, and glutaraldehyde-Hb molar ratio (GDA/Hb). Each factor included 3 levels which were 6.4, 6.9 and 7.4 for PH value, 4%, 6% and 8% for Hb concentration, and 10:1, 12:1 and

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14:1 for GDA : Hb. The temperature for the reaction was 4 ± 0.5. The polymeric rate and high-M.W. molecule (>600KD)—medium-sized molecule (120KD ~ 600KD) ratio were detected by HPLC at 2hr and 4hr after beginning of the reaction.

The result suggested that, when detected at 4 hr since beginning of the reaction, the sequence of magnitude of the effect of the condition factors on polymeric rate is GDA/Hb > PH > [Hb], that on output of high-M.W. molecules was GDA/Hb > PH > [Hb], and that on output of medium-sized-molecules was GDA/Hb > [Hb] > PH; the optimum combination of the factors as the polymeric rate was concerned was: [Hb] = 8%, PH = 7.4, GDA/Hb = 12:1; and when PH value decreases, the output of ultra-high-M.W. molecule was hibited. Based on above, we adopted higher concentration of Hb and cross linker (GDA) to raise the polymeric rate, and lower PH value and addition of competitive inhibiting agent (lysine) to decrease the production of ultra-high-M.W. molecules. Thus after repeated experiments, an ultimate condition was selected where Hb concentration is 12%, GDA/Hb is 12:1, PH value is 6.7, and lysine is added at 5:1 of lys/Hb, reaction time is within 2.5hr temperature is 4.0 ± 0.5.The result of the reaction under this condition is showed in the figure and the table.

Polymeric rate	67.7 ± 0.2%
Composition (%)	
32KD	32.3 ± 0.2
64KD	18.0 ± 1.3
128-670KD	36.2 ± 4.2
>670KD	10.5 ± 3.6

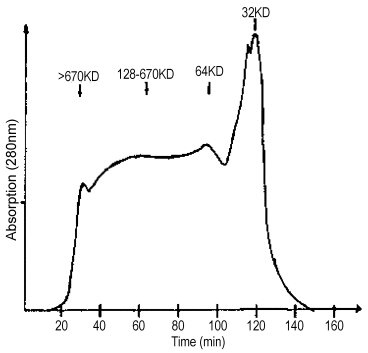

Figure 1:M.W. distribution of Poly-Hb(Analysis Condition:Pharmacia FPLC; Column:Sephacryl S300; Mobil Phase:0.02MPBS+0.1MNacl; Flow Rate: 30ml/hr;Detecror:280nm,0.05Sens.)

Second Generation Pegylated Hemoglobins for the Validation of New Paradigms for the Design of HB Based Oxygen Carriers (HBOCS)

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The new paradigms for the design of HBOCs are: 1) enhanced molecular size of Hb, 2) high O_2 affinity and 3) high solution viscosity and oncotic pressure. A

thiolation-mediated maleimide chemistry based PEGylation protocol that was developed by us has generally validated these new paradigms. This protocol increases the O_2 affinity but allows for systematic tuning of the colligative properties of HBOCs. To determine optimal O_2 affinity for HBOC efficacy, we are generating second generation PEGylated Hbs with a wide spectrum of O_2 affinities.

One approach is based on reductive alkylation of low O_2 affinity Hb with PEG aldehyde, targeted to the α-amino groups of Hb. This new protocol has been used for the PEGylation of [3-Phospho, 2-hydroxypropyl-Val-1(β)]$_2$-HbA. The latter is derived by the modification of HbA with glyceraldehyde-3-phosphate and exhibits both functional and spectroscopic signatures of a Hb with covalently bound DPG at its ββ-cleft. Reductive alkylation of HbA or this low O_2 affinity Hb with PEG-20K aldehyde generates molecular species with an unaltered O_2 affinity as compared to the parent protein. The PEGylated Hb with 2 copies of PEG-20K chains/Hb tetramer, exhibits an enhanced molecular size comparable to that of (SP-PEG-5K)$_6$-HbA, a non-hypertensive HBOC that is entering phase I clinical trial. Thus this new PEGylation protocol represents a very promising approach for systematically generating PEG-Hbs having varied O_2 affinities that could expose the interplay of Hb O_2 affinity and colligative properties in neutralizing Hb-induced vasoactivity.

New Pegylated Human Hemoglobins for Evaluation as Oxygen Carrying Plasma Expanders

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The solutions of PEG-Hb can be considered as O2 carrying plasma expanders (OCPE) since their colligative properties are comparable to that of plasma expanders. These PEG-Hb solutions are vasoinactive. Reducing the auto-oxidation of the PEG-Hb represents the next challenge in developing Hb based O2 carriers (HBOC) with increased shelf life. Targeting the PEGylation of Hb to sites that reduces the auto-oxidation is an obvious approach. Identification of such sites requires mapping of the potential reactive sites of Hb and needs the development of simple, PEG-functionalizing approaches that target the PEG chains on to the functional groups of Hb in a site-specific fashion. Towards this goal, p-isocyanato, phenyl iso-thiocyanate (ITC) and m,p-di-isocyanato, phenyl ITC designed as functionalizing moieties to target the PEGylation to the ?-amino groups of Hb have been synthesized. On reaction of these with PEG, a urethane linkage is generated between the PEG and the isocyanate of phenyl ITC, thereby functionalizing PEG with ITC. Reaction of HbA (0.5 mm) in PBS buffer pH 7.4, with a 20 fold molar excess of ITC-phenyl-PEG-5K at 23oC modifies Hb at its four ?-amino groups. This P5K4-HbA has an O2-affinity slightly higher than HbA, but is comparable to P5K6-HbA, and has a molecular radius of 5.2 nm. The selectivity of the ITC-phenyl-PEG to the ?-amino groups of Hb has been used to generate PEG-HbA with a mass of 40 K of PEG [P10K4-HbA and

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P(5 + 5)K4-HbA] using either ITC-phenyl-PEG 10K or ITC phenyl m,p-bis - PEG-5K. These PEG-Hbs are useful additions to the class of PEGylated Hbs that can serve as OCPEs.

Post-hemodilution Blood Viscosity Influences the Effects of αα-HB on Hemodynamics and Vascular Hindrance in Rats

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To discriminate between pharmacological and rheological effects of αα-Hb, we compared the impact of blood/αα-Hb mixtures with high vs. low viscosity on hemodynamics and vascular hindrance (VH). VH, determined as the ratio between peripheral resistance (PR) and blood viscosity measured at 128.5 s^{-1}, is an absolute indicator of vascular tone and is therefore more relevant than PR measurements alone in models of experimental hemodilution. Anesthetized rats were subjected to 50% exchange transfusion (ET) with 1) high viscosity (Hv) solutions: whole blood (WB, n = 5; Hct = 0.40 L/L) or WB mixed with αα-Hb (Hb-Hv, n = 5; Hct = 0.40 L/L) or with 2) low viscosity (Lv) solutions: αα-Hb (Hb-Lv, n = 5; Hct = 0.20 L/L) or human albumin (alb, n = 5; Hct = 0.20 L/L). For 2 hours after ET, mean arterial pressure (MAP), heart rate (HR), aortic blood flow (ABF), PR and VH were monitored. Hemodynamics and VH remained unchanged in animals transfused with WB. RP decreased immediately after infusion of alb, as a result of increased ABF and decreased MAP, while VH remained unchanged. Hb-Lv induced an immediate and sustained increase of VH (more than 200%) despite unchanged PR. Conversely, in Hb-Hv animals, VH increase was delayed and of lesser extent (40 to 148%) while PR increased gradually (maximum increase after 2 hours, 94%). Taken together these data demonstrate the beneficial effects of associating αα-Hb with WB to maintain physiological viscosity and limit vasoconstriction due to pharmacological properties of Hb.

A Model of Nitric Oxide Distribution in Arterioles in the Presence of Hemoglobin-Based Blood Substitutes

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Scavenging of endothelium-derived nitric oxide (NO) by hemoglobin-based oxygen carriers (HBOC) is considered one of the major causes of vasoconstriction

following application of HBOC. High NO reactivity of free Hb and extravasation of Hb may be determinants of this effect. To gain a quantitative understanding of NO interactions with HBOC in arterioles, we developed a detailed theoretical model of NO diffusion that considers: 1) reactions of NO with free Hb and red blood cell (RBC) Hb in the arteriolar lumen and the surrounding capillaries and in the interstitial space between the endothelial and smooth muscle cells when Hb molecules extravasate; 2) RBC-free layer in the arteriolar lumen; 3) shear stress dependent NO production by the endothelium; 4) NO reaction with oxygen; 5) NO reaction with myoglobin in the parenchymal cells. The model takes into account different reaction rates for free Hb and that enclosed in the RBC. We vary the HBOC reactivity with NO and the degree of extravasation. The parameters of the model are based on available experimental data. The model predicts a significant dependence of NO concentration within the arteriolar smooth muscle on NO-Hb binding rate and degree of Hb extravasation. The changes occur within the range of activation of soluable guanylate cyclase in the smooth muscle cells.

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Oxygen Transport in the Capillaries, Microvascular Networks, and Organs by Hemoglobin-Based Blood Substitutes

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The major purpose for using hemoglobin-based oxygen carriers (HBOC) is to enhance oxygen delivery to tissue. In both design of new substitutes and clinical applications it is important to know how the oxygen transport processes depend on HBOC affinity, cooperativity, and concentration under different conditions. We developed oxygen transport models at the capillary, microvascular network, and organ levels. At the capillary level, we estimated intravascular resistance to oxygen transport as a function of HBOC characteristics. We then utilized these values in a detailed microvascular network model where we consider a tissue volume containing tens or hundreds of capillary segments. We also considered a compartmental model that we apply to a whole organ. These models are used to make predictions of intravascular and tissue PO_2 under conditions of physiological importance, such as ischemia, and hypoxic and anemic hypoxia following HBOC transfusion. The model allows assessments of the effects of HBOC affinity and cooperativity in relation to these

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parameters in the RBCs, as well as HBOC concentration. The models can be used to assist in design of HBOC and in interpretation of results of clinical trials.

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