Intramolecular Cross-linking of Beta Subunits and PEGylation of Bovine Stroma Free Hemoglobin For Use as a Hemoglobin Based Oxygen Carrier

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Hemoglobin based oxygen carriers (HBOCs) have the potential characteristics to fill the clinical needs that are not met by normal blood transfusions. The raw material sources for HBOC come from an untapped supply of human or bovine blood, which can eliminate the issue of shortages and blood type complications. This characteristic applicable in treatment of individuals in remote locations or even used to battlefields where emergency hospitals may be hundreds of miles away. By combining know methods of hemoglobin (Hb) modifications, we were able to create a novel HBOC known as BXLHb (Bovine Xeno Low Hemoglobin). BXLHb is BPEGXLHb 0.0 subunits then further modifications on surface residues using 540s molecular weight (MW) polyethylene glycol (PEG) to produce a bovine polyethylene glycol cross-linked hemoglobin (BPEGXLHb). This new HBOC was characterised and studied for its potential uses in oxygen transporting solutions. Evidence shows that the combination of methods did provide the final product with structural stability as well as oxidative stress and limit the formation of superoxides.

**OBJECTIVE**

We hope to address the challenges of availability, immunologic response, the short yet stable half-life, increased cost, altered oxidative stress, and enhance vectoring properties. The issue of stability was made apparent from post research that observed Hb is a substantially less stable than that of the 0 subunits and tends to dissociate from the tetramer structure. Resolution to this issue was intermolecular cross-linking a subunits which increase the stability of the HBOC. Using well established methods of cross-linking human Hb in subunits and altering it for cross-linking bovine HB complexes was done by changing the initial conformational structure of deoxyHb to oxyHb. The aim of this research was to determine if cross-linking the more stable 0 subunits will have greater stability for the overall HBOC intermolecular cooperation stability.

Unmodified cell free Hemoglobin (CFH) or stroma free hemoglobin (SFHb) was well as iron releasing reagents for cross linking and solution properties of the PEGylated Hb does provide a conformational stability, can be used as oxidative scavengers to decrease in stability of BPEGXLHb under UV-Vis light to avoid the oxidative stress. The evidence shows that BPEGXLHb has a higher stability of HBOC under UV-Vis light. Further modifications to increase antioxidant capability coupled with the wide range of different methods of PEGylation, BPEGXLHb has the potential to be a functional stable HBOC.

**REFERENCES**

Nagababu, E., & Rifkind, J. M. (2000). *Analytical Chemistry Anal. Chem.*, 78(13), 4634-4642. *Reaction of Hydrogen Peroxide with Ferrylhemoglobin: Initial Kinetics using UV-Vis spectrometry, but EPR and Fe4+ release did show that BPEGXLHb had a higher attenuation of hydrogen peroxide and produce less Fe4+. This slight decrease in stability of BPEGXLHb under UV-Vis is acceptable, due to the gain in vascular retention of the increase Mw from the conjugation of the 3.7G PEG. This data provided evidence that bovine Hb-β subunit cross-linking prior to PEGylation provided a notable degree of conformational stability, but did not provide much protection for the Fe for the oxidative stress challenge. The evidence shows that PEGylated Hb-β subunit cross-linking does provide a conformational stability, can attenuate oxidative stress and reduce potential toxicity from ROS. BPEGXLHb also provides improved vascular retention and increased oxidative stress due to the increased Mw from the PEG chains. Further modification to increase antioxidant capability coupled with the wide range of different methods of PEGylation, BPEGXLHb has the potential to be a functional stable HBOC.

**ACKNOWLEDGEMENTS**

MS-APS Program Director Dr. Michelle McBegon My Mentor and Thesis Committee Chair Dr. Li Ma My Thesis Committee: Dr. Hans-Joachim Schans and Dr. Jonathan Abruamula Georgia Southern University Department of Physics and Chemistry Chairs: Dr. Clayton Heller and Dr. John DeCeasaro PEGylated B Subunit Intra-Molecular Cross-linked Bovine Hemoglobin Based Oxygen Carrier Blood Substitutes By Gil Salazar

**MATERIALS & METHODS**

**Methods of Characterisation**

- SELC to determine molecular size distribution using running buffer was 50mM Mops, with flow rate of 0.3mL/min, sample Load of 100ng on a Superose 12 column.
- MALDI-ToF MS to determine molecular weight distribution using a matrix 3,3'-Dimethoxy-4,4'-dihydroxybenzidine (DHB) acid, solvant of 0.1% trifluoroacetic acid (TFA), sample to matrix solvent ratio was 50:1 and scan the 200-1600 Da mass for stainless steel plate.
- UV-Vis Absorbance Spectroscopy to determine oxidation states and protein concentration

**Methods of Studying Oxidative Stress under Hydrogen Peroxide Challenge**

- Initial Kinetics using different molar concentrations of HbH2O2 1:0, 400, 750, 900 and 1050

**Characterisation Results**

- SELC Results
- MALDI-ToF MS Results

**Materials**


**Preparations**

- PUF for HBOC elution in 6% in Mops at pH 7.4.
- Induced whole blood state using 10%.
- Cross flow filtration with saline at 38 ° C.
- Cross flow filtration with saline at 38 ° C with mBr of 3.4 hours.
- Cross flow filtration with saline at 38 ° C with mBr of 3.4 hours.
- Cross flow filtration with saline at 38 ° C with mBr of 3.4 hours.
- Cross flow filtration with saline at 38 ° C with mBr of 3.4 hours.

**Characterization Results**

- UV-Vis Results
- LDS-PAGE Results

**Materials & Methods**

**Vis Results**

**Characterization Results**

- Flow cytometry
- Western Blot
- MALDI-ToF Mass Spectral Table

**Oxidative Stress under H2O2, Study Results**

**Initial Kinetics using UV-Vis**

**MALDI-ToF Mass Spectral Table**

**Conclusion**

Blood substitutes that are stable and functional have been sought after for many years and recently HBOCs have been one of the forefront. Although the intended substitute is not completely replace the need for whole blood, it is often misunderstood due to the word substitute. A more semantically realistic term would be artificial blood substitutes. Understanding the purpose of HBOCs we can begin to realise the advantages and limitations of application. From there new methods can be developed to either enhance the advantages or tackle the limitation issues. HBOC should be a more convenient method to prevent whole blood shortage temporarily provide similar oxygen carrying capability, maintain systemic pressure, and reduce oxidative stress. The two main focus of this thesis was the prepared and characterization of bovine β subunit cross-linking and PEGylation. Both modifications shown significant improvement in stability and functional properties. The main focus of this thesis was the prepared and characterization of bovine β subunit cross-linking and PEGylation. Both modifications shown significant improvement in stability and functional properties.

**REFERENCES**


Zhang, X., Wang, H., & Wu, B. (2014). *Advanced Drug Delivery Reviews,* 75(7), 1588-1601. *Polymerized with Poly(ethylene glycol) linked Hb does provide a conformational stability, can increase oxidative scavengers and alter the oxidative stress due to the increased Mw from the PEG chains. Further modification to increase antioxidant capability coupled with the wide range of different methods of PEGylation, BPEGXLHb has the potential to be a functional stable HBOC.*