THE DEVELOPMENT OF POLYHB-FG: A NOVEL BLOOD SUBSTITUTE WITH THE POTENTIAL TO SUPPORT COAGULATION

by

Naomi Sie-Wan Wong

Department of Biomedical Engineering

McGill University, Montreal

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For SDR, for changing my life without meaning to.

Abstract

Polyhemoglobin (polyHb) is one of the most promising blood substitutes in development. It is awaiting approval from the FDA and has already been approved for routine surgical use in South Africa. Though it has been successful as an oxygen carrier, we have shown in the present study that with the lack of platelets and coagulation factors in the current polyHb formulations, there could be a risk of coagulation problems when large volumes of polyHb are administered. We therefore develop a novel blood substitute that would solve potential coagulation problems in a hemodiluted setting, while still being able to support oxygen transport. We developed polyhemoglobin-fibrinogen (polyHb-Fg), by crosslinking hemoglobin and fibrinogen with glutaraldehyde. Our *in vitro* tests showed that platelet aggregation was not potentiated by the addition of polyHb or polyHb-Fg. Using *in vitro* whole blood coagulation tests, hemodilution with polyHb adversely delayed the clotting mechanism. On the other hand a formulation of polyHb-Fg was able to achieve similar clotting times as whole blood, even with hemodilution. Thus, this formulation of polyHb-Fg has the potential for applications in the infusion of large volumes of blood substitute without interfering with coagulation.

Resumé

La polyhémoglobine (polyHb) est l'un des substituts du sang les plus prometteurs à l'étude. Ce composé attend l'approbation de la FDA et a déjà été approuvé pour l'usage chirurgical courant en Afrique du Sud. Bien que la polyHb ait connu un succès comme porteur de l'oxygène, nous avons montré dans la présente étude que le manque de plaquettes et des facteurs de coagulation dans les formulations courantes de polyHb, il pourrait y avoir un risque de problèmes de coagulation quand de grands volumes de polyHb sont administrés. Nous développons donc un nouveau substitut du sang qui résoudrait les problèmes potentiels de coagulation dans une situation d'hémodilution, tout en continuant d'assurer le transport de l'oxygène. Nous avons développé le polyhémoglobine-fibrinogène (polyHb-Fg), en réticulant l'hémoglobine et le fibrinogène avec du glutaraldéhyde. Nos essais *in vitro* ont prouvé que l'agrégation de plaquette n'a pas été augmentée par l'addition du polyHb ou du polyHb-Fg. Lors d'essais *in vitro* de coagulation de sang entier, l'hémodilution avec le polyHb retarde défavorablement le mécanisme de coagulation. D'autre part une formulation de polyHb-Fg permet d'obtenir un temp de coagulation semblable à celui du sang entier, même en cas d'hémodilution. Ainsi, cette formulation de polyHb-Fg a le potentiel requis pour des applications dans l'injection de grands volumes de produits de remplacement du sang sans interférer avec la coagulation.

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Abbreviations

%	percent
<	less than
>	greater than
μ	micro
Ω	ohms
AR	aggregation rate
$CaCl_2$	calcium chloride
CAT	catalase
dL	decilitre
FAM	Fibrinogen-coated Albumin Microcapsule
Fg	fibrinogen
g	gravitational force
g	grams
G	gauge
glut	glutaraldehyde
h	hours
Hb	hemoglobin
HR	high resolution
IU	international unit
K	$* 10^3$
kD	kiloDalton
kg	kilogram
L	litre
lys	lysine
M	molar
m	metre
m	milli
mg	milligram

min	minutes
mOsmal	milliosmolar
MWCO	molecular weight cut-off
n/a	not available
nm	nanometers
^{o}C	degrees Celsius
polyHb	polyhemoglobin
polyHb-Fg	polyhemoglobin-fibrinogen
polyHb-SOD-CAT	polyhemoglobin-(superoxide dismutase)-catalase
polyHb-Tyr	polyhemoglobin-tyrosinase
RBC	red blood cell
RL	Ringer's Lactate
RPM	rotations per minute
s	seconds
SC	sodium citrate
SFHb	stroma-free hemoglobin
SOD	superoxide dismutase
t	temperature
T	time
UV	ultraviolet
V_E	elution volume
V_o	void volume
vs	versus

Preface

The thesis is composed of four sections. The first section is a general introduction and literature review that provides background information pertaining to the thesis. The third section includes a general discussion of my thesis. The final section is a general summary and conclusions. An appendix at the end also includes additional methods used in the course of my research and preliminary results. For the second section, I have taken advantage of the option provided by section C of the "Thesis preparation and submission guidelines" for a manuscript-based thesis which states:

> 1. Candidates have the option of including, as part of the thesis, the text of one or more papers submitted, or to be submitted, for publication, or the clearly-duplicated text (not the reprints) of one or more published papers. These texts must conform to the "Guidelines for Thesis Preparation" with respect to font size, line spacing and margin sizes and must be bound together as an integral part of the thesis.
>
> 2. The thesis must be more than a collection of manuscripts. All components must be integrated into a cohesive unit with a logical progression from one chapter to the next. In order to ensure that the thesis has continuity, connecting texts that provide logical bridges preceding and following each manuscript are mandatory.
>
> 3. The thesis must conform to all other requirements of the "Guidelines for Thesis Preparation" in addition to the manuscripts. The **thesis must include** the following: a table of contents, a brief abstract in both English and French, an introduction which clearly states the rational and objectives of the research, a comprehensive review of the literature (in addition to that covered in the introduction to each paper), a final conclusion and summary, a thorough bibliography. Appendix containing an ethics certificate in the case of research involving human or animal subjects, microorganisms, living cells, other biohazards and/or radioactive material.
>
> 4. As manuscripts for publication are frequently very concise documents, where appropriate, additional material must be provided (e.g., in appendices) in sufficient detail to allow a clear and precise judgement to be made of the importance and originality of the research reported in the thesis.

5. In general, when co-authored papers are included in a thesis the candidate must have made a substantial contribution to all papers included in the thesis. In addition, the **candidate is required to make an explicit statement in the thesis as to who contributed to such work and to what extent**. This statement should appear in a single section entitled "Contributions of Authors" as a preface to the thesis. The supervisor must attest to the accuracy of this statement at the doctoral oral defence. Since the task of the examiners is made more difficult in these cases, it is in the candidate's interest to clearly specify the responsibilities of all the authors of the co-authored papers.

6. When previously published copyright material is presented in a thesis, the candidate must include signed waivers from the publishers and submit these to the Graduate and Postdoctoral Studies Office with the final deposition, if not submitted previously. The candidate must also include signed waivers from any co-authors of unpublished manuscripts.

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8. **In no case can a co-author of any component of such a thesis serve as an external examiner for that thesis**.

In this thesis, a manuscript of an original paper is presented in the second section and will be submitted for publication. It has its own Abstract, Introduction, Materials and Methods, Results, Discussion, and References. The results that are not included in the manuscript are described in the appendix at the end of the thesis.

Contributions of authors of manuscripts to be published:

Section 2 is a manuscript to be published. I am the first author. I performed the experiments and analysed the results. The other author is my thesis supervisor Dr.

T.M.S. Chang, who offered the research planning and guidance. There is no other author involved.

Introduction

General blood substitute history

The development of hemoglobin-based red blood cell substitutes has had increased interest over the last few decades (1, 2). Their success has been propelled by the recognition of the need for an abundant, safe and reliable blood supply. With the current shelf-life of donated blood being 42 days (3), there will always be the potential for a blood shortage, especially in times of crisis. The risks of HIV and hepatitis infection from blood transfusions have never been lower as the blood supply is at its safest (4). There are still, however, emerging risks that include West Nile Virus, SARS, variant Creutzfeldt-Jakob disease as well as non-viral risks like bacterial contamination (4-6). Human error in blood collection, labeling and transfusion also adds to the risk in conventional blood transfusion (6).

Hemoglobin based blood substitutes address many of the shortcomings of the current blood supply. They can be sterilized and stored in a dry state, thereby increasing the shelf-life to over a year and they eliminate the bacterial and viral risk for transfusions (7-9). Because there are no elements of the red blood cell membrane remaining, there is no need for cross matching and typing when a transfusion is administered. They have been tested for use in many applications especially for perioperative conditions, anemia, and trauma (9-12). More applications will emerge as the research in this exciting area continues.

Hemoglobin is a 64.5 kD protein that consists of two α and two β polypeptide chains, each attached to a heme group that is capable of binding one oxygen molecule (2, 12). It is a tetramer that is stable within the membrane of the red blood cell, but once free from the cell and circulating throughout the body, it quickly deteriorates into dimers that are toxic, thereby being removed from the circulation and excreted in the urine. In order to prevent this dissociation from occurring, various modifications on the hemoglobin molecule have been explored in order to make the molecule more stable. These include polymerization, conjugation, intramolecular crosslinking, and encapsulation in liposomes or other biodegradable materials (2, 9, 13). Liposome-encapsulated hemoglobins have been formulated with increased circulation times of around 48h (14). Hemoglobin has been recombinantly produced using yeast or *E. coli* to have the two α units of the dimers fused (13). Advantages would be that the hemoglobin could be produced in a way that is well-characterized with a more homogeneous molecular structure and did not depend on human or animal sources (13). A recently developed blood substitute based on recombinant human hemoglobin has been shown to perform as well in heterologous blood for resuscitation after perioperative blood loss without causing pulmonary hypertension (15). A combination of polymerization and conjugation has been used to create a high molecular weight hypo-oncotic oxygen carrier that can be used as a blood additive, when used alone, or can be administered with a plasma expander when being used to replace blood volume (10). Nanocapsules made from biodegradable materials have been investigated to contain hemoglobin and other enzymes to increase the stability and effectiveness of the hemoglobin (1, 16).

Polyhemoglobin and its current status

One of the most promising hemoglobin-based blood substitutes is hemoglobin crosslinked with glutaraldehyde in a polymerization reaction (17). This reaction crosslinks hemoglobin molecules intermolecularly to form polyhemoglobin (polyHb). This has been extensively characterized and developed in various animal models and has shown no serious adverse events in clinical trials within a broad spectrum of patients (18). Applications include trauma surgery, surgical procedures that require large amounts of red blood cell transfusions, and preoperative administration to save autologous blood (19).

There are currently three companies that are developing polymerized hemoglobin, two with successful results. Biopure Corporation polymerizes bovine hemoglobin with the crosslinker glutaraldehyde to form HBOC-201. In a study of patients undergoing infrarenal aortic reconstruction, HBOC-201 significantly eliminated the need for any allogenic red blood cell transfusion in 27% of the patients and was well tolerated (20). Although the overall reduction of red blood cell transfusion was not significantly different, HBOC-201 did delay the red blood cell transfusions in all patients, suggesting that it can serve as an oxygen bridge until red blood cells are available. Another study showed similar results for patients undergoing cardiac surgery (21). Biopure is currently producing blood substitutes that have been approved for surgical use in South Africa and are awaiting FDA approval of a Biological License Application. They also have a similar product used in veterinary medicine for the treatment of anemic dogs (16). Northfield Laboratories also uses glutaraldehyde as the crosslinker, but human hemoglobin prepared

from outdated blood instead to make PolyHeme. Polyheme has been shown to be as effective as red blood cells in maintaining the total hemoglobin concentration in acute blood loss with no adverse side effects (22). A recent study gave 171 patients rapid infusion of 1-20 units of PolyHeme to massively bleeding patients. When compared to patients who declined blood transfusion for religious reasons, it significantly reduced the 30-day mortality rate (23). Hemosol Inc. uses a different crosslinker, o-raffinose, to polymerize hemoglobin also isolated from outdated human blood. They recently stopped their phase III clinical trials for cardiac bypass grafting surgery due to increased cardiac effects in the blood substitute group.

PolyHb has also been modified for other applications. Polyhemoglobin-superoxide dismutase-catalase (polyHb-SOD-CAT) has been developed for use in ischemia-reperfusion conditions (11). Superoxide dismutase (SOD) and catalase (CAT) are enzymes normally found within the red blood cell. Together, they help to convert superoxide into hydrogen peroxide, which is subsequently converted into water and carbon dioxide. In the absence of these protective enzymes, there could be increased ischemia-reperfusion injury (1). PolyHb-SOD-CAT has shown that it can reduce the formation of methemoglobin during preparation, and stabilize the crosslinked Hb resulting in decreased oxidative iron and heme release (16). It has also been shown to supply oxygen to ischemic tissues without causing reperfusion injury in the transient global brain ischemia-reperfusion model injury in rats (24).

Polyhemoglobin-Tyrosinase (polyHb-Tyr) has been developed as a treatment for melanoma. The lowering of tyrosine can inhibit the growth of melanoma in culture. Promising results were found with the tyrosinase maintaining its enzymatic activity even after crosslinking with glutaraldehyde while the polyHb-Tyr could still act as an oxygen carrier to supply oxygen for radiation therapy (25, 26). In both cases, polyHb-SOD-CAT and polyHb-Tyr have shown that additional functionalities can be successfully added to polyHb without affecting the oxygen carrying capabilities of polyHb.

Hemostatic limitations of PolyHb

Currently, polyHb as a blood substitute will only replace the function of the red blood cell as an oxygen carrier. Blood has many different components with different physiological functions (18). With high blood volume loss and subsequent replacement with blood substitute, oxygen transport may be recovered, but the cause of the blood loss may be still present without an adequate concentration of platelets and coagulation factors to act at the site(s) of blood loss (27). Thus, the blood loss could theoretically continue when a large volume of blood substitute is administered. Those with hemostatic disorders may be more prone to high volume blood loss; polyHb could act as a bridge providing oxygen transport until a suitable type of whole blood is available, but will not be able to act as the final treatment or would have to be administered in conjunction with something else in order to stop the bleeding.

Platelet transfusions are available for the prevention and treatment of thrombocytopenia (28-31). Donated platelets have an even shorter shelf-life than red blood cells, 5-7 days,

and often are pooled with other donations to give one transfusion, increasing the exposure to the recipient (3, 5, 29, 32). There has been much research done to prolong platelet storage, including cryogenic storage, cold storage, photochemical treatment and lyophilization (5, 30, 32, 33). Others have worked on the development of platelet substitutes, which are either platelet-derived or non-platelet-derived. Table 1 shows a summary of the work that has been published up to date in the area of platelet substitutes. Many have incorporated platelet glycoproteins or fibrinogen into the platelet substitute in order to interact with the platelets to induce platelet activation, secretory activity and aggregation (28, 30, 34).

Much of the developing platelet substitutes are still years away from clinical use (3, 30). There are many issues that stall their development, such as the agreement of the appropriate tests to measure the efficacy of platelet substitute and a defined endpoint for clinical trials (3, 35). The platelet substitutes currently under development would all be limited for use in thrombocytopenic situations. The most promising platelet substitute to date, Synthocytes™, are of micron dimensions, which as learned from the development of blood substitutes, would be quickly cleared from the circulation (9, 28, 36). If suitable platelet substitutes and coagulation factors can be developed, their combination with a red blood cell substitute would allow for the better treatment of advanced hemorrhagic shock in the future (12).

Product	Platelet derived	Diameter	Shelf life	Duration of hemostatic activity	Mechanism of action	Effects	Status	Reference
Infusible platelet membranes	yes	~0.6 µm	2-3 years at 2-8°C	24 h	promotes local procoagulant activity, may actively bind to the subendothelium	reduced bleeding time, but short effect	phase II trials completed; phase III are contemplated	30-32, 37-39
RBC with surface bound RGD peptides - Thromboerythrocytes	no	8 µm	n/a	0	interacts with activated platelets; cross-links to and coaggregates with platelets rather than cause platelet aggregation; binds specifically to GPIIb/IIIa	some have shown shortened bleeding times, but for most, no hemostatic efficacy *in vivo*	development stopped	30, 32, 35, 37, 40
RBC with surface bound fibrinogen	no	8 µm	n/a	n/a	enhances platelet aggregation	shortened bleeding times; no hemostatic efficacy *in vivo*	development stopped	39-41
Fibrinogen-coated albumin microcapsules (FAM) - Synthocytes	no	3.5-4.5 µm	n/a	3 h	enhances adhesion of platelet containing aggregates to activated endothelial cells, mediated by the GPIIb/IIIa receptor	shortens bleeding time in rabbits; promising *in vitro* results with human platelets	still preclinical	28, 30, 33, 42
FAM - Thrombospheres	no	1.2 µm	n/a	72 h	cross-links to and coaggregate with platelets rather than cause platelet aggregation	shortened bleeding times in rabbit; enhanced agonist-induced platelet aggregation	still preclinical	30, 37, 41
Liposomes - Plateletosomes	no	200-750 nm; 200-300 nm	n/a	n/a	no effects *in vitro*	shortened bleeding times in rats	still preclinical	30, 43, 44
Rehydrated, lyophilized platelets	yes		n/a	9.5 min half-life; 4-6 h	similar hemostatic properties as normal platelets; some metabolic properties (spreading)	liposomes interacted with platelets *in vitro*; capable of supporting thrombin generation, promising *in vivo* effects	no clinical trials yet	32, 39-41, 45, 46
Platelets produced *in vitro*	yes	2-4 µm	n/a	n/a		megakaryocytic cell line that expresses GPIIb/IIIa	no further testing as of yet	47
Platelet microparticles or fragments	yes	~0.6 µm	3				phase I trials completed; phase II results promising	38, 41

Table 1: Platelet substitutes under development

Aims and objectives

Hemolink™, a polyHb, was found not to affect the activation and function of human platelets in whole blood *in vitro* (27). Another polyHb also has no adverse effect on the complement system and platelet count *in vivo* (48). It has also previously been shown to not induce or enhance agonist induced platelet aggregation (27, 49). HBOC-201, another polyHb, has been evaluated using *in vitro* tests to assess its effects on coagulation testing methods (50). All of the methods used platelet rich plasma, which with HBOC-201 takes on a reddish colour instead of colourless. Mechanical clot detectors were less sensitive than optical clot detectors to the presence of the HBOC-201. In general, it found that up to a threshold concentration, there were no effects on the results. Impedance aggregometry showed no induced platelet aggregation or modified patterns of aggregation when agonists were added (51). There are, however, contradicting results found by others (27, 52, 53). PolyHb was found to not activate platelets but potentiated agonist induced platelet aggregation using a microplate reader (54). Similar results were found with agonist-induced platelet aggregation using impedance and light transmittance aggregometry (52, 53). *In vivo*, polyHb was found to shorten the prolonged bleeding time and decrease blood loss from ear incisions in rabbits (52).

While there have been extensive studies on the effects of polyHb on platelets and coagulation, little is known about these effects as a result of hemodilution. We wanted to further study the effects of polyHb on platelets and coagulation in a hemodiluted setting comparable to the replacement of a large volume of polyHb. In the following chapters, we describe a novel blood substitute prepared by polymerizing hemoglobin with

fibrinogen to form polyhemoglobin-fibrinogen (polyHb-Fg). We will compare the effects of polyHb-Fg with polyHb on whole blood coagulation and platelet aggregation.

References

1. Chang TMS. Future generations of red blood cell substitutes. Journal of Internal Medicine. 2003, 253: 527-535.
2. Chang TMS. Blood Substitutes: Principles, Methods, Products and Clinical Trials Volume I, Karger Landes systems, 1997
3. Lapierre V, Herve P. Perspectives. La Presse Medicale. 1999, 28: (4), 1336-1340.
4. Busch MP, *et al.* Current and emerging infectious risks of blood transfusions. JAMA. 2003, 289: (8), 959-961.
5. Corash L. New technologies for the inactivation of infectious pathogens in cellular blood components and the development of platelet substitutes. Bailliere's Best Practice and Research in Clinical Haematology. 2000, 13: (4), 549-563.
6. Goodnough LT, *et al.* Transfusion medicine: looking to the future. The Lancet. 2003, 361: 161-169.
7. Chang TMS. Modified hemoglobin-based blood substitutes: crosslinked, recombinant and encapsulated hemoglobin. Vox Sanguinis. 1998, 74: (supp 2), 233-241.
8. Kjellstrom BT. Blood substitutes: where do we stand today?. Journal of Internal Medicine. 2003, 253: 495-497.
9. Chang TMS. Future prospects for artificial blood. Trends in Biotechnology. 1999, 17: 61-67.
10. Barnikol WKR, *et al.* New artificial oxygen carriers made of pegulated polymerised pyridoxylated porcine haemoglobin (P4Hb). Comparative Biochemistry and Physiology Part A. 2002, 132: 185-191.
11. D'Agnillo F, Chang TMS. Polyhemoglobin-superoxide dismutase-catalase as a blood substitute with antioxidant properties. Nature Biotechnology. 1998, 16: 667-671.
12. Moore EE. Blood substitutes: the future is now. Journal of American College of Surgeons. 2003, 196: (1), 1-17.

13. Winslow RM. Current status of blood substitute research: towards a new paradigm. Journal of Internal Medicine. 2003, 253: 508-517.
14. Phillips WT, *et al.* Polyethylene glycol-modified liposome-encapsulated hemoglobin: a long circulating red cell substitute. The Journal of Pharmacology and Experimental Therapeutics. 1999, 288: 665-670.
15. Malhotra AK, *et al.* Resuscitation with a novel hemoglobin-based oxygen carrier in a swine model of uncontrolled perioperative hemorrhage. The Journal of Trauma Injury, Infection, and Critical Care. 2003, 54: 915-924.
16. Chang TMS, *et al.* Two future generations of blood substitutes based on polyhemoglobin-SOD-catalase and nanoencapsulation. Advanced Drug Delivery Reviews. 2000, 40: 213-218.
17. Chang TMS. Stabilisation of enzymes by microencapsulation with a concentrated protein solution or by microencapsulation followed by cross-linking with glutaraldehyde. Biochemistry and Biophysics Research Communications. 1971, 44: (6), 1531-1536.
18. Chang TMS. Blood Substitutes: Principles, Methods, Products and Clinical Trials Volume II. Publisher: Karger Landes systems. 1997, 235 pages-.
19. Izumi Y, *et al.* Evaluation of the capabilities of a hemoglobin vesicle as an artificial oxygen carrier in a rat exchange transfusion model. ASAIO Journal. 1997, 43: 289-297.
20. LaMuraglia GM, *et al.* The reduction of the allogenic transfusion requirement in aortic surgery with a hemoglobin-based solution. Journal of Vascular Surgery. 2000, 31: (2), 299-308.
21. Levy JH, *et al.* Polymerized bovine hemoglobin solution as a replacement for allogeneic red blood cell transfusion after cardiac surgery: results of a randomized, double-blind trial. The Journal of Thoracic and Cardiovascular Surgery. 2002, 124: (1), 35-42.
22. Gould SA, *et al.* The first randomized trial of human polymerized hemoglobin as a blood substitute in acute trauma and emergent surgery. Journal of the American College of Surgeons. 1998, 187: 113-122.

23. Gould SA, *et al*. The life-sustaining capacity of human polymerized hemoglobin when red cells might be unavailable. Journal of the American College of Surgeons. 2002, 195: 445-455.
24. Powanda DD, Chang TMS. Cross-linked polyhemoglobin-superoxide dismutase-catalase supplies oxygen without causing blood-brain barrier disruption or brain edema in a rat model of transient global brain ischemia-reperfusion. Artificial Cells, Blood Substitutes, and Immobilization Biotechnology. 2002, 30: (1), 23-37.
25. Yu BL, Chang TMS. *In vitro* enzyme kinetics of microencapsulated tyrosinase. Artificial Cells, Blood Substitutes and Immobilization Biotechnology. 2002, 30: (5&6), 533-546.
26. Yu BL, Chang TMS. Tyrosinase artificial cells for the removal of systemic tyrosine in rats. p76 in Canadian Federation of Biological Societies (CFBS) 45th Annual Meeting, Montreal, Quebec, Canada, June 2002.
27. Leytin V, *et al*. Hemolink, an o-raffinose cross-linked haemoglobin-based oxygen carrier, does not affect activation and function of human platelets in whole blood *in vitro*. British Journal of Haematology. 2003, 120: 535-541.
28. Davies AR, *et al*. Interactions of platelets with Synthocytes, a novel platelet substitute. Platelets. 2002, 13: 197-205.
29. Vaickus L, *et al*. Platelet transfusion and alternatives to transfusion in patients with malignancy. Stem Cells. 1995, 13: 588-596.
30. Lee DH, Blajchman MA. Platelet substitutes and novel platelet products. Expert Opinion on Investigational Drugs?. 2000, 9: (3), 457-469.
31. Chao FC, *et al*. Infusible platelet membrane microvesicles: a potential transfusion substitute for platelets. Transfusion. 1996, 36: 536-542.
32. Blajchman MA. Platelet substitutes. Vox Sanguinis. 2000, 78: (supl 2), 183-186.
33. Blajchman MA. Novel platelet products, substitutes and alternatives. Transfusion Clinical Biology. 2001, 8: 267-271.
34. Agam G, Livne AA. Erythrocytes with covalently bound fibrinogen as a cellular replacement for the treatment of thrombocytopenia. European Journal of Clinical Investigation. 1992, 22: 105-112.

35. Alving BM, *et al.* Frozen platelets and platelet substitutes in transfusion medicine. Transfusion. 1997, 37: 868-876.
36. Keipert PE, Chang TMS. Preparation and *in vitro* characteristics of a blood substitute based on pyridoxylated polyhemoglobin. Applied Biochemistry and Biotechnology. 1984, 10: 133-141.
37. Silberman S. Platelets: preparations, transfusion, modifications, and substitutes. Archives of Pathology & Laboratory Medicine. 1999, 123: 889-894.
38. Galan AM, *et al.* Infusible platelet membranes improve hemostasis in thrombocytopenic blood: experimental studies under flow conditions. Transfusion. 2000, 40: (9), 1074-1080.
39. Blajchman MA. Novel platelets and substitutes. Fifth Congress of the European Haematology Association (educational book). 2000, 110-113.
40. Blajchman MA, Lee DH. Novel platelet products and substitutes. Transfusion Medicine Reviews. 1998, 12: (3), 175-187.
41. Rivera J, Vicente V. Potential and current development of platelet products. Haematologica (educational book). 1999, 84: 115-119.
42. Levi M, *et al.* Fibrinogen-coated albumin microcapsules reduce bleeding in severely thrombocytopenic rabbits. Nature Medicine. 1999, 5: (1), 107-111.
43. Galan AM, *et al.* Preparations of synthetic phospholipids promote procoagulant activity on damaged vessels: studies under flow conditions. Transfusion. 1998, 38: 1004-1010.
44. Kitaguchi T, *et al.* Characterization of liposomes carrying von Willebrand factor-binding domain of platelet glycoprotein Ibalpha: a potential substitute for platelet transfusion. Biochemical and Biophysical Research Communications. 1999, 261: 784-789.
45. Fiscer TH, *et al.* Splenic clearance mechanisms of rehydrated, lyophilized platelets. Artificial Cells, Blood Substitutes, and Immobilization Biotechnology. 2001, 29: (6), 439-451.
46. Nishiya T, *et al.* Platelet interactions with liposomes carrying recombinant platelet membrane glycoproteins or fibrinogen: approach to platelet substitutes. Artificial

Cells, Blood Substitutes, and Immobilization Biotechnology. 2001, 29: (6), 453-464.

47. Chang TMS, *et al.* Two future generations of blood substitutes based on polyhemoglobin-SOD-catalase and nanoencapsulation. Advanced Drug Delivery Reviews. 2000, 40: 213-218.
48. Ning J, Chang TMS. Effects of homologous and heterologous stroma-free hemoglobin and polyhemoglobin on complement activation, leucocytes and platelets. Journal of Biomaterials, Artificial Cells and Artificial Organs. 1990, 18: (2), 219-232.
49. Lalla FR, *et al.* Effects of pyridoxylated polyhemoglobin and stroma-free hemoglobin on ADP-induced platelet aggregation. Journal of Biomaterials, Artificial Cells and Artificial Organs. 1989, 17: (3), 363-369.
50. Jahr JS, *et al.* Effects of hemoglobin glutamer-250 (bovine) (HBOC-201, Hemopure) on coagulation testing. American Journal of Therapeutics. 2002, 9: 431-436.
51. Toussaint M, *et al.* Hemoglobin-based oxygen carriers do not alter platelet functions: study of three chemically modified hemoglobin solutions. Intensive Care Medicine. 2003, 29: 62-68.
52. Lee DH, *et al.* o-Raffinose cross-linked hemoglobin improves the hemostatic defect associated with anemia and thrombocytopenia in rabbits. Blood. 2000, 96: (10), 3630-3636.
53. Lieberthal W, *et al.* O-Raffinose cross-linking markedly reduces systemic and renal vasoconstrictor effects of unmodified human hemoglobin. The Journal of Pharmacology and Experimental Therapeutics. 1999, 288: (3), 1278-1287.
54. Mondoro TH, *et al.* Hemoglobin A_0 and α-crosslinked hemoglobin (α-DBBF) potentiate agonist-induced platelet aggregation through the platelet thromboxane receptor. Artificial Cells, Blood Substitutes, and Immobilization Biotechnology. 1998, 26: (1), 1-16.

Manuscript

Polyhemoglobin-Fibrinogen: a Novel Blood Substitute with the Potential to Support Coagulation in a Hemodiluted Setting

Naomi S.W. Wong and Thomas M.S. Chang

Artificial Cells & Organs Research Centre
Departments of Biomedical Engineering, Physiology and Medicine
Faculty of Medicine, McGill University

Abstract

Polyhemoglobin (polyHb) is currently being assessed in phase III trials under various formulations. At present, none contain clotting factors or platelet substitutes to aid in hemostasis. A novel blood substitute, polyhemoglobin-fibrinogen (polyHb-Fg), was studied and compared to polyHb for its effect on platelet aggregation and coagulation. Platelet aggregation was tested using a whole blood aggregometer and coagulation was assessed through whole blood clotting time. Hemodilution of whole blood with polyHb showed corresponding reductions in platelet aggregation. Varying formulations of polyHb-Fg and polyHb showed no significant differences in platelet aggregation measurements and did not induce platelet aggregation to the extent that whole blood did. One formulation of polyHb-Fg showed similar clotting times as whole blood whereas the paired polyHb showed significantly higher clotting times. This formulation of polyHb-Fg has the potential to be a more beneficial blood substitute in applications of severe hemorrhagic shock.

Introduction

Polyhemoglobin (polyHb) has been developed as a blood substitute for over a decade (1,2). It is currently approved in South Africa for use in surgeries and is expecting more approvals for further use. PolyHb is a blood substitute that is sterilizable, with a more than 1 year shelf-life at room temperature, and eliminates the need for cross-matching and type (3-5). Though the blood supply has never been safer, there will continue to be new and emerging risks, like West Nile virus and variant Creutzfeldt-Jakob disease, which are cause for concern (6). PolyHb offers multiple benefits compared to blood by offering a safe and limitless alternative. Current applications are grouped into three areas: perioperative applications, acute hemorrhagic shock, and regional perfusion (7).

As an oxygen carrier, polyHb is one of the most advanced in the area. Blood, however, is a multifunctional fluid (8). As a blood substitute, polyHb is limited by its lack of platelets and/or coagulation properties. In situations of high blood volume loss, dilutional coagulopathy could occur when large amounts of polyHb are administered (9). PolyHb would only be able to act as a bridge to whole blood transfusion as the platelets and coagulation factor concentrations in the polyHb-transfused recipient may not be sufficient for primary hemostasis. There has been development in platelet substitutes to combat thrombocytopenia, but with limited success (10,11).

PolyHb has been studied for its effects on platelet activation and aggregation, with conflicting results. PolyHb has been found to potentiate aggregation of rabbit platelets

induced by collagen, but not by ADP. Though the mechanism is unknown, it is suggested that the hemostatic defect is improved through enhanced platelet reactivity and/or induced vasoconstriction (12). Another group found that ADP did increase aggregation of human, gel-filtered platelets (13). On the other hand, polyHb has been found to not affect activation and function of human platelets in whole blood (9). It has also been shown to not enhance platelet aggregation using rat platelet rich plasma (14). Discrepancies may be due to differences in the methods of measurement (9).

The aim of this study was not to design a treatment for thrombocytopenia, but to develop a PolyHb that can be used for severe hemodilution so that it can deliver oxygen to tissues while being able to reduce the potential for excessive bleeding. This study describes the development of a novel blood substitute, polyhemoglobin-fibrinogen (polyHb-Fg), which is capable of having similar clotting times as whole blood. PolyHb and polyHb-Fg were compared by measuring their effects on platelet aggregation and whole blood coagulation.

Materials and Methods

Platelet aggregation was measured using an impedance aggregometer (Chrono-log Corp, Model 500). This measures the change in impedance between two electrode wires placed in the sample as an agonist is added. Blood coagulation was measured through clotting times, where a test tube filled with the sample is periodically tilted until a clot is visibly formed.

Materials

Glutaraldehyde (glut), 25% aqueous solution, bovine fibrinogen, and sodium citrate solution were purchased from Sigma Company. Lysine (lys) was purchased from Fisher Scientific. All other reagents were of analytical grade.

Preparation of polyHb and polyHb-Fg

Stroma-free hemoglobin (SFHb) was prepared as previously described (15). Briefly, whole bovine heparinized blood (from the McGill Animal Resource Centre) was centrifuged and washed with saline three times to separate the plasma proteins, white blood cells and platelets from the red blood cells. The red blood cell solution was then lysed with a sodium phosphate buffer (15mOsmal, pH 7.4) in order to release the hemoglobin. The stroma was separated from the Hb by two toluene extractions. The final Hb solution was then dialysed (14000 MWCO, Spectropor) against saline or Ringer's lactate solution before further use (Baxter).

PolyHb was prepared as previously described (16), with modifications. Briefly, 10 mL of SFHb was mixed on an orbital shaker at 170 RPM with a 1.3M lysine solution in a molar ratio of 10:1 lysine to hemoglobin at 5°C. Degassed, 25% aqueous glutaraldehyde was added in four equal aliquots over a period of 15 minutes in glutaraldehyde to hemoglobin molar ratio ranging from 16:1 to 24:1.

PolyHb-Fg was prepared in a similar way as polyHb. Various amounts of bovine fibrinogen, ranging from 5-350mg, were dissolved in 4 mL of either sterile 0.9% sodium chloride injectable or Ringer's lactate. Fibrinogen was dissolved into the SFHb before polymerization was started, or added to the reaction mixture 4 or 20 hours after polymerization began.

After 24 hours of polymerization, for both polyHb and polyHb-Fg, the reaction was stopped by quenching with 2.0M lysine solution in a molar ratio of 200:1 lys to Hb. The solutions were then dialysed against a Ringer's lactate solution overnight. Some samples were concentrated by using Centriprep centrifugal devices (MWCO 50K, Microcon). Samples were spun at 1500g for intervals of 20 minutes at room temperature until the final Hb concentrations of the polyHb/polyHb-Fg pair were equal. The solutions were sometimes filtered before further aggregation experiments.

Variations on mixing speeds, ratio, and other parameters were performed as necessary. PolyHb and polyHb-Fg were prepared in pairs with identical parameters for comparison.

Aggregation Experiments

Blood was collected from male Sprague Dawley rats (Charles River), 180-350g via cardiac puncture with a 22G needle (Becton Dickenson). Vacutainers containing 0.129M of sodium citrate (Becton Dickenson) were used to ensure a constant 1:9 ratio (anticoagulant:blood) by volume. The rats were anesthetized with an intraperitoneal injection of sodium pentobarbital (MTC Pharmaceuticals, 65mg/kg). After the blood was collected, the rats were sacrificed via lethal injection and/or cervical dislocation.

Previously prepared samples were tested in matching polyHb and polyHb-Fg pairs. Samples were placed in polypropylene cuvettes (Chrono-log Corporation) with small magnetic stirrers in the bottom (Payton). Five hundred microliters (μL) of saline was first put into the cuvette followed by 200μL of citrated blood and 300μL of the blood substitute. A control sample of 500μL saline and 500μL citrated blood was always used as the first sample in order to confirm the integrity of the blood. The samples were prewarmed to 37°C in the warming stations of the aggregometer. With the cuvette in the sample well, the electrode was placed in the cuvette and allowed to reach a baseline. Once stable, 3.5μL of bovine collagen (Chrono-log) was added to the stirred sample. The impedance was recorded on a chart recorder (Kipp & Zonen BD 40) until the rate of aggregation (change in impedance) was no longer increasing. The rate of aggregation was recorded as the maximum slope in the impedance vs time chart. Measurements were performed in triplicate, when possible.

Clotting Time Experiments

Blood was collected from male Sprague Dawley rats, 180-350g, via cardiac puncture. 5mL syringes (Becton Dickenson) containing no anticoagulant were used with 20G needles (Becton Dickenson). The rats were anesthetized with an intraperitoneal injection of sodium pentobarbital (65mg/kg) and sacrificed once the experiment was complete via lethal injection and/or cervical dislocation.

Paired samples of polyHb and polyHb-Fg were used for comparisons. Glass tubes were prepared with 250μL and 400μL of blood substitute and labeled. Two hundred and fifty microliter aliquots of fresh blood were added to the 250μL aliquots of blood substitute. One hundred microliter aliquots of fresh blood to the 400μL aliquots of blood substitute. The timing was started when the fresh blood is added. The tubes were left untouched for one minute, after which the tubes were tilted 80° and back to check for clot formation. Clotting time was defined as the point at which the blood no longer flows from its position when inverted (17). If the sample had not yet clotted, tubes were tilted in a similar manner every 10s thereafter. If 2 minutes had gone by without clot formation, the tubes were tilted every 30s. After 10 minutes, tubes were left alone and checked once more before the samples were discarded after 20 minutes. Once a clot had formed, the time was noted as well as a description of the clot. If a clotting time was not reached, the time was recorded as ">20min". Measurements were performed in triplicate.

Determination of hemoglobin concentration

Hemoglobin (Hb) concentration was measured by spectrophotometric analysis with "Total Hemoglobin Kit" from Sigma-Aldrich.

Molecular weight distribution

Microsep™ centrifugal devices (Pall) were used to determine a general molecular weight distribution. Samples were diluted 10-fold with saline and placed in the 1000K MWCO device. Samples were spun at 5000g and 4°C for 8-50 minutes, until the level in each compartment of the centrifugal device remained stable. The portion that passed through the filter was put into a 300K MWCO device and spun at similar conditions. The volumes and Hb concentrations of each portion were used to calculate the molecular weight distribution of the sample.

Statistical analysis

Clotting time data was analysed using the Wilcoxon rank sum test. All other data was analysed with an unpaired student t-test. Differences are defined as statistically significant if $p < 0.05$. All figures show error bars of one standard error.

Results

Aggregation Experiments

Effects of dilution of blood by polyHb

To first show the effect of polyHb on platelet aggregation, polyHb [10:1 lys to Hb, 16:1 glut to Hb, [Hb] = 7.76g/dL] was mixed with blood in the following ratios (μL polyHb/μL blood) by volume: 0/500, 100/400, 200/300, 250/250, 300/200, 400/100, 500/0. PolyHb was citrated with sodium citrate (0.129M) in a 1:9 ratio (sodium citrate:blood) by volume. Results are shown in figure 1. Platelet aggregation decreased as the proportion of polyHb to blood increased. The decrease was considerable at the first level tested and continued in a proportional manner. Only single measurements were performed. Two other formulations of polyHb were also compared and showed similar results (Figure 2,3).

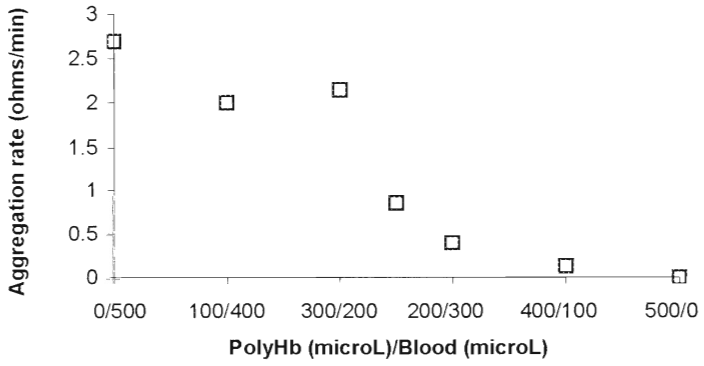

Figure 1: Polyhemoglobin dilution series - 24h crosslink. Dilution series of polyHb prepared with 5:1 lys, 17:1 glut ratios, 0.22 μm filtered, [Hb] = 7.76g/dL

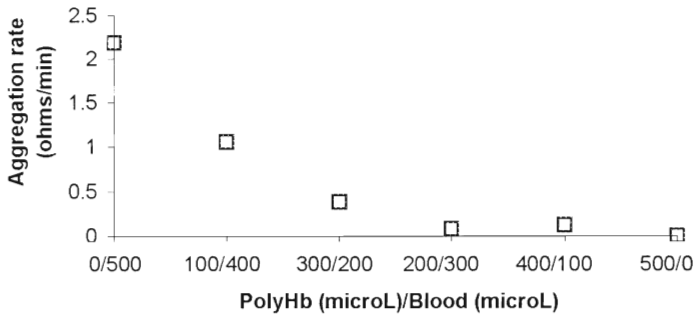

Figure 2: Polyhemoglobin dilution series - 48h crosslink. Dilution series of polyHb prepared with 5:1 lys, 17:1 glut ratios, 0.22 μm filtered, [Hb] = 10g/dL

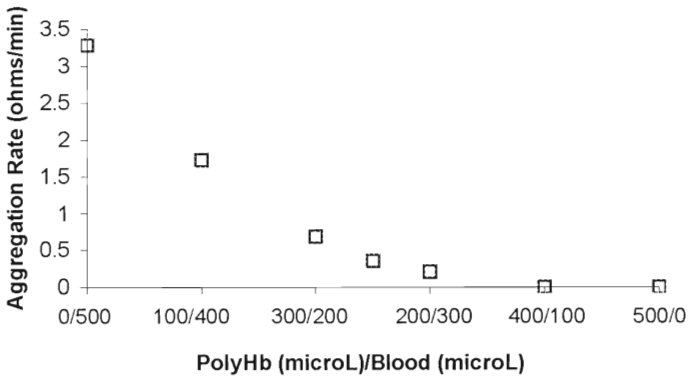

Figure 3: Polyhemoglobin dilution series - experimental sample. Dilution series of polyHb prepared with 10:1 lys, 16:1 glut ratios, unfiltered, [Hb] ~ 7g/dL

Effects of amounts of fibrinogen in polyHb-Fg

A polyHb-Fg solution was prepared by the addition of a fibrinogen solution (500-8750g/dL) to the reaction mixture after 20 hours of crosslinking and continued for another 4 hours (10:1 lys, 24:1 glut). Twenty, 45, 90, 175, or 350mg of dissolved fibrinogen were added to the polymerizing Hb as described in the methods section. The aggregation rates relative to the aggregation rate of blood were compared (Figure 4). In order to normalize data taken from different experiments, the aggregation rates (AR) of the samples were divided by the AR of the control sample of whole blood. This ratio is used for the value plotted on the y-axis. The filtered polyHb-Fg solutions (8μm-filter) consistently aggregated at a higher rate than the unfiltered polyHb-Fg solutions.

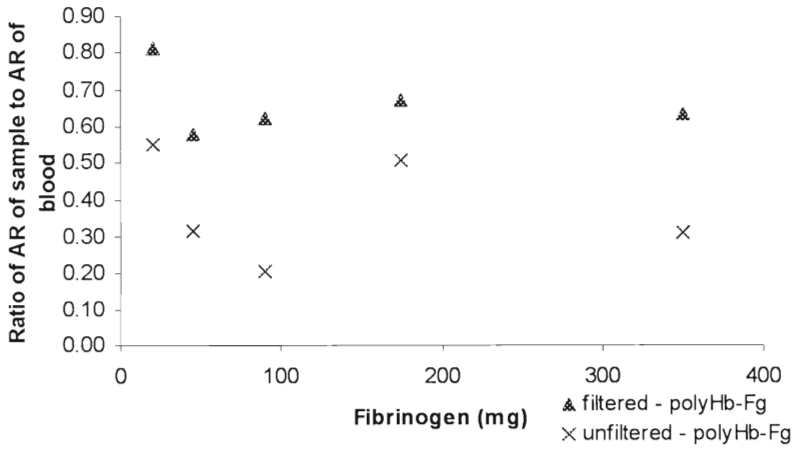

Figure 4: Aggregation rates of polyHb-Fg - different levels of fibrinogen. Different formulations of polyHb-Fg were prepared with a matching formulation of polyHb (10:1 lys, 24:1 glut).

At lower fibrinogen levels, 20 and 40mg added at the beginning of crosslinking (10:1 lys, 20:1 glut), there were no significant differences (Figure 5). At these lower levels, the fibrinogen would dissolve more easily than in previous samples and no fibrinogen aggregates were observed, however, the aggregation rates between polyHb and polyHb-Fg did not differ significantly.

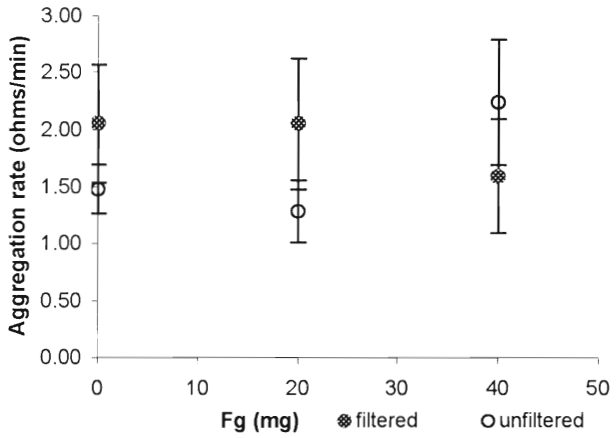

Figure 5: Aggregation rates of polyHb (Fg = 0mg) and polyHb-Fg - lower levels of fibrinogen. Samples with 10:1 lys, 20:1 glut and different levels of fibrinogen added at the beginning of crosslinking. 8μm filtered, [Hb] ~7.5g/dL. Figure shows average ± SEM

Effects of fibrinogen solution solvent

Two fibrinogen solutions were made, 90 mg in 4mL of saline and 90mg in 4mL of Ringer's lactate. The solutions were added to the reaction mixture after 20 hours of crosslinking and continued for another 4 hours (10:1 lys, 24:1 glut). The aggregation rates relative to the aggregation rate of blood were compared (Figure 6). There were no significant differences between polyHb and polyHb-Fg for either fibrinogen in saline or fibrinogen in Ringer's lactate.

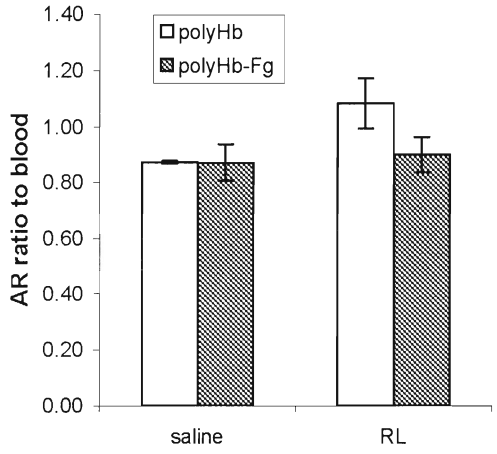

Figure 6: Comparison of fibrinogen solution solvents. Samples were 10:1 lys, 24:1 glut with 90mg of fibrinogen in 4mL of Ringer's lactate or saline added at T=20h, [Hb] ~ 5.6 g/dL. Figure shows average ± SEM

Effects of anticoagulant

Another series of polyHb and polyHb-Fg (10:1 lys, 20:1 glut) was prepared with 5, 10, 20, and 40mg of fibrinogen added at the beginning of the crosslinking reaction. Heparin was also used as an anticoagulant for the rat blood for comparison (72 IU for 5mL blood). There was an overall increase in the rate of aggregation for whole blood using heparin. No significant differences in aggregation rate, however, were observed between polyHb and any of the polyHb-Fg samples (Figure 7).

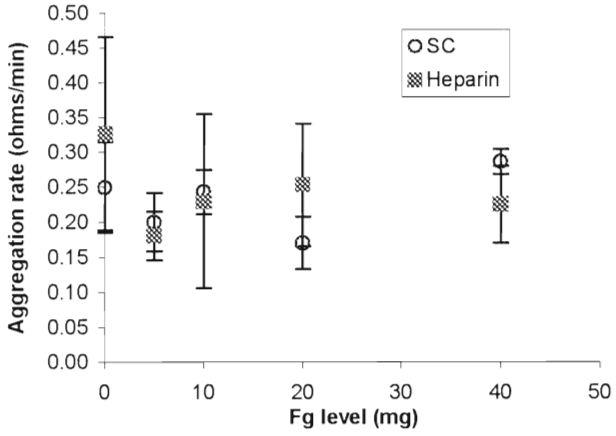

Figure 7: Aggregation rates with low fibrinogen levels and heparinized blood. Comparison of aggregation rates when blood is anticoagulated with heparin or with sodium citrate. 10:1lys, 20:1 glut with fibrinogen added at the beginning of crosslinking. All unfiltered, [Hb] ~9.3g/dL. Figure shows average ± SEM

Effects of glutaraldehyde levels

PolyHb-Fg was prepared with a 10:1 lys to Hb ratio but the glut ratio to 1 Hb ranged from 19.7-20.3 in 0.1 increments. Forty milligrams of fibrinogen was added before the crosslinking began. A single polyHb sample was prepared (10:1 lys, 20:1 glut). There was no trend observed in aggregation rates with increasing glut ratios in the polyHb-Fg samples (Figure 8). There were also no significant differences in the ratio of the aggregation rates of the samples over whole blood between polyHb and any of the polyHb-Fg samples.

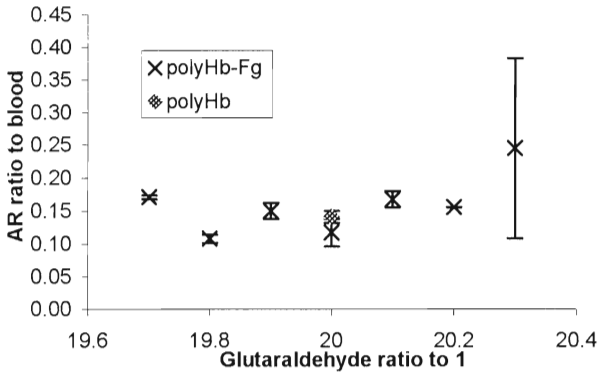

Figure 8: Comparison of polyHb and polyHb-Fg - different glut ratios. Glut ratio ranged from 19.7-20.3 with 0.1 increments for polyHb-Fg. PolyHb had a constant glut ratio of 20:1. 40mg of fibrinogen added at the beginning of crosslinking with 10:1 lys. All unfiltered, [Hb] ~9.3g/dL. Figure shows average ± SEM

Comparing at higher glut ratios, additional paired samples of polyHb and polyHb-Fg were prepared with lys ratio of 10:1 and glut ratios of 22:1 and 23:1. 40mg of fibrinogen were added at the beginning of crosslinking. With a sample size of 3 measurements, there were no significant differences between polyHb and polyHb-Fg at each different glut ratio, shown in Figure 9 ($p = 0.053$ and 0.063, respectively). There were, however, significant differences between the polyHb-Fg samples at different glut ratios. The polyHb-Fg 20:1 sample had a significantly higher aggregation rate than the polyHb-Fg 22:1 and 23:1 sample ($p = 0.02$ and 0.03, respectively).

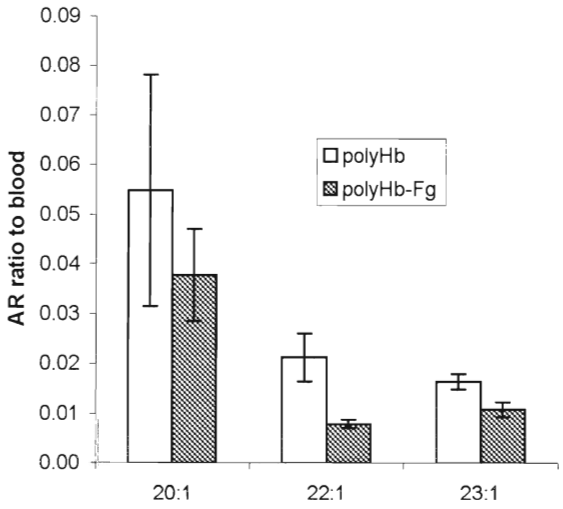

Figure 9: Comparison of polyHb and polyHb-Fg - higher glut ratios. 40mg of fibrinogen added at the beginning of crosslinking, 10:1 lys ratio All unfiltered, [Hb] ~9g/dL. Figure shows average ± SEM

Effects of time of fibrinogen addition to reaction mixture

Paired samples of polyHb and polyHb-Fg were prepared with 20:1 lys and 10:1 glut ratio. 40mg of fibrinogen was added either before polymerization to SFHb or after 4 hours of crosslinking in a Ringer's lactate solution to the reaction mixture. There were no significant differences observed in the rate of aggregation between the two (Figure 10). By adding fibrinogen after the beginning of polymerization, there will be dilution of the sample due to the added volume of liquid, making Hb concentrations higher in samples where fibrinogen is added at the beginning of polymerization compared to when it is added in the middle of polymerization.

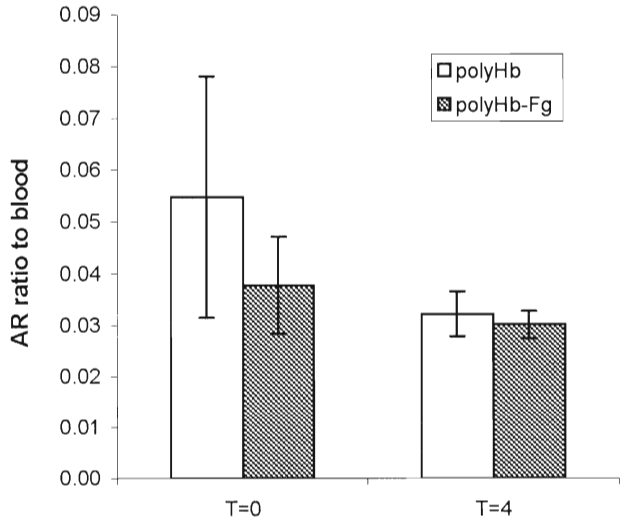

Figure 10: Comparison of time of addition of fibrinogen to polymerization. 10:1 lys, 20:1 glut with 40mg of fibrinogen added at the beginning of crosslinking for T=0 and 40mg dissolved in 4mL of Ringer's lactate for T=4. All unfiltered, $[Hb]_{T=0}$ ~9g/dL, $[Hb]_{T=4}$ ~7.2g/dL. Figure shows average ± SEM

Figure 11 shows the comparison of two samples, one with 40mg of fibrinogen added at the beginning of the polymerization reaction and the other with 40mg of fibrinogen dissolved in 4mL of Ringer's lactate added after 20h crosslinking. The lys ratio was 10:1 and the glut ratio was 24:1. With a sample size of 3 measurements each, there were no significant differences between polyHb and polyHb-Fg in both cases ($p = 0.135$ and 0.185, respectively).

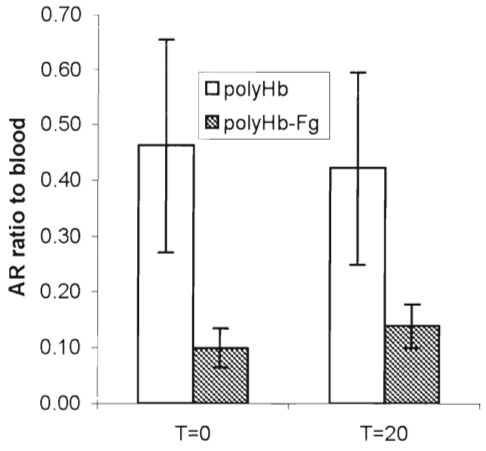

Figure 11: Comparison of fibrinogen addition time. Unfiltered samples with 10:1 lys, 24:1 glut with 90mg of fibrinogen dissolved in 4mL of Ringer's lactate added t either T=20h or at the beginning of crosslinking. $[Hb]_{T=0}$ ~7g/dL, $[Hb]_{T=4}$ ~6g/dL. Figure shows average ± SEM

Effects of temperature of reaction

The temperature of the reaction mixture was increased to 37°C from 5 °C to ensure that the fibrinogen was completely dissolved. With a 10:1 lys, 20:1 glut ratio, and 40mg of fibrinogen dissolved directly into the warmed SFHb, no significant differences were found between polyHb and polyHb-Fg (Figure 12). The sample prepared at 37°C was extremely thick when compared to previous samples prepared at 5°C.

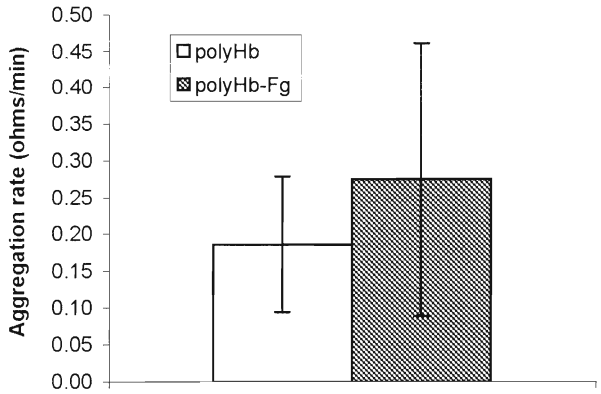

Figure 12: Aggregation rates at 37°C. 10:1 lys, 20:1 glut with 40mg added at the beginning of crosslinking. All samples unfiltered. Figure shows average ± SEM

Clotting Time Experiments

Of all of the samples tested for aggregation, only one sample showed interesting results in the clotting time experiments. PolyHb and polyHb-Fg with 10:1 lys, 20:1 glut, and 40mg fibrinogen in 4mL of Ringer's lactate added after 4 hours of crosslinking showed markedly different results (Table 2). There were significant differences between the polyHb and polyHb-Fg samples. With polyHb, the clots that formed did not adhere to the glass tubes and no clotting time could be assessed. On the other hand, all of the clots that formed for polyHb-Fg stuck to the walls of the glass tube and could be quantified with a clotting time ($p<0.01$). Figure 13 shows the concentration dependence of the clotting time for polyHb-Fg. The polyHb samples still had not clotted completely at similar times as the polyHb-Fg samples according to the definition of the end point for clot formation (17). The polyHb tends to form partial clots that do not adhere to the glass tube.

PolyHb			PolyHb-Fg		
Clot Formation (min)	Adhesion	Comment	Clot Formation (min)	Adhesion	Comment
2.67	yes		>20	no	2.5 floating clot
>20	no	6.67 sliding clot	3.75	yes	
>20	no	5.17 sliding clot	5	yes	
>20	no	2 sliding clot	2	yes	
>20	no	2 sliding clot	2	yes	
>20	no	2 sliding clot	2	yes	
>20	no	>10 sliding clot	9.67	yes	
9.40	yes		>10	yes	
>20	no	6.07 sliding clot	>10	yes	

Table 2: Clotting times with fibrinogen added at T = 4h. Clotting time measurements for 400μL of blood substitute to which 100μL of fresh blood is added

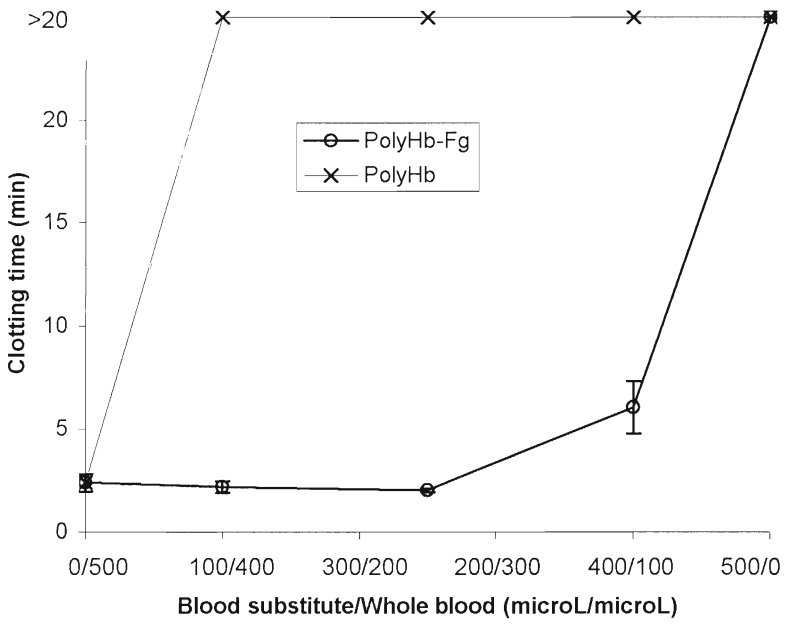

Figure 13: Clotting times comparison when T = 4h. 10:1 lys, 20:1 glut with 40mg of fibrinogen dissolved in 4mL Ringer's lactate added at T=4h. All samples unfiltered, [Hb] ~7.2g/dL. $p < 0.01$. Figure shows average ± SEM

Molecular weight distribution

Two samples were used to determine a general molecular weight distribution. The results are shown in table 3.

	22:1, T = 0		20:1, T = 4	
Molecular weight	PolyHb*	PolyHb-Fg*	PolyHb	PolyHb-Fg
>1000kD	29%	87%	0%	60%
>300kD, <1000kD	50%	6%	100%	40%
<300kD	7%	1%	0%	0%

* some Hb stuck on membrane and so totals do not equal 100%

Table 3: Molecular weight distributions. The comparison of the molecular weight distributions of polyHb and polyHb-Fg with 10:1 lys and 40mg of fibrinogen added either at the beginning or in 4mL Ringer's lactate after 4 hours

Discussion

PolyHb has been successfully used as a blood substitute in clinical trials involving mostly perioperative uses (1). In cases of extreme hemorrhagic shock, large amounts of infused polyHb would result in extreme hemodilution with no platelets or coagulation factors. This may interfere with coagulation at the site of injury. In this study, we have shown that polyHb-Fg, a modified form of polyHb, was able to maintain similar clotting times as whole blood whereas polyHb could not.

Not all formulations worked and often, as for the case of all aggregation measurements, there were no significant differences between paired polyHb and polyHb-Fg samples. Various formulations were tried, including differing levels of cross-linker and fibrinogen, different times of addition as well as Fg dissolved in saline or Ringer's lactate. None showed any significant differences between the pairs in aggregation rate. PolyHb-Fg may be too small to have a measurable effect on platelet aggregation, as many platelet substitutes are of micron dimensions (10, 18). Liposomes carrying platelet glycoproteins or fibrinogen have been developed with a smaller diameter of 200-700 nm. Though they showed no *in vitro* effects, they shortened the bleeding times in rats (19-20). Various formulations of polyHb-Fg may show similar effects.

Other developments of platelet substitutes also use fibrinogen to induce interactions with platelets via the GPIIb/IIIa receptor (10, 21-23). Fibrinogen has been crosslinked with glut and was found to be still able to polymerize into fibrin, which is essential for clot formation (24). Previous attempts for a platelet substitute used fibrinogen conjugated to

red blood cells, but due to their relative size, they stayed close to the center of the vessel instead of closer to the walls where platelet aggregation is necessary (10). Synthocytes™ are 3.5-4.5 μm in diameter (25); platelets are approximately 2-4 μm in diameter (26). Despite this similarity, Synthocytes™ will likely be quickly removed from the circulation. There may be a compromise of size and lifespan since for cases of thrombocytopenia, large platelet substitutes can contribute more readily to the platelet plug formation.

For the clotting time measurements, only one formulation showed significant differences. This may be attributed to the time of addition of the fibrinogen solution. When fibrinogen is added at the beginning of the reaction, it will be incorporated throughout the interior and exterior of the polyHb-Fg complex. When it is added in the middle of the polymerization, it will be excluded from the middle of the complex as the hemoglobin will have already begun polymerizing together. The clotting test results suggest that this way, fibrinogen may be more concentrated on the exterior of the polyHb-Fg particles and may play a larger role in platelet coagulation. Polymerizing for a long time may also end up internalizing the fibrinogen, since for 40mg added to the reaction mixture, the molar ratio is ~130:1 (Hb:fibrinogen). The effect of increasing the amount of fibrinogen that is added in order to increase the exterior fibrinogen concentration may be limited by fibrinogen's solubility and the increased viscosity of the reaction mixture.

The action of polyHb-Fg largely depends on the physiology of the blood in the recipient. The clotting factors must still be present, though the concentration may be diluted by the

blood substitute added. Further *in vitro* testing and *in vivo* characterization should be pursued with the successful polyHb-Fg.

Acknowledgments

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References

1. Chang TMS. Future generations of red blood cell substitutes. Journal of Internal Medicine. 2003, 253: 527-535.
2. Chang TMS. Recent and future developments in modified hemoglobin and microencapsulated hemoglobin as red blood cell substitutes. Artificial Cells, Blood Substitutes, and Immobilization Biotechnology. 1997, 24: (1&2), 1-24.
3. Chang TMS. Modified hemoglobin-based blood substitutes: crosslinked, recombinant and encapsulated hemoglobin. Vox Sanguinis. 1998, 74: (supp 2), 233-241.
4. Kjellstrom BT. Blood substitutes: where do we stand today? Journal of Internal Medicine. 2003, 253: 495-497.
5. Chang TMS. Future prospects for artificial blood. Trends in Biotechnology. 1999, 17: 61-67.
6. Busch MP, *et al.* Current and emerging infectious risks of blood transfusions. JAMA. 2003, 289: (8), 959-961.

7. Moore EE. Blood substitutes: the future is now. Journal of American College of Surgeons. 2003, 196: (1), 1-17.
8. Chang TMS. Blood Substitutes: Principles, Methods, Products and Clinical Trials Volume II. Publisher: Karger Landes systems. 1997, 235 pages.
9. Leytin V, *et al.* Hemolink, an o-raffinose cross-linked haemoglobin-based oxygen carrier, does not affect activation and function of human platelets in whole blood *in vitro*. British Journal of Haematology. 2003, 120: 535-541.
10. Lee DH, Blajchman MA. Platelet substitutes and novel platelet products. Expert Opinion on Investigational Drugs. 2000, 9: (3), 457-469.
11. Lapierre V, Herve P. Perspectives. La Presse Medicale. 1999, 28: (4), 1336-1340.
12. Lee DH, *et al.* o-Raffinose cross-linked hemoglobin improves the hemostatic defect associated with anemia and thrombocytopenia in rabbits. Blood. 2000, 96: (10), 3630-3636.
13. Lieberthal W, *et al.* O-Raffinose cross-linking markedly reduces systemic and renal vasoconstrictor effects of unmodified human hemoglobin. The Journal of Pharmacology and Experimental Therapeutics. 1999, 288: (3), 1278-1287.
14. Lalla FR, *et al.* Effects of pyridoxylated polyhemoglobin and stroma-free hemoglobin on ADP-induced platelet aggregation. Journal of Biomaterials, Artificial Cells and Artificial Organs. 1989, 17: (3), 363-369.
15. Chang TMS, Prakash S. Procedures for microencapsulation of enzymes, cells and genetically engineered microorganisms. Molecular Biotechnology. 2001, 17: 249-260.
16. Powanda DD, Chang TMS. Cross-linked polyhemoglobin-superoxide dismutase-catalase supplies oxygen without causing blood-brain barrier disruption or brain edema in a rat model of transient global brain ischemia-reperfusion. Artificial Cells, Blood Substitutes, and Immobilization Biotechnology. 2002, 30: (1), 23-37.
17. Lee RI, White PD. A clinical study of the coagulation time of blood. The American Journal of Medical Sciences. 1913, 145: 495-503.
18. Blajchman MA. Novel platelets and substitutes. Fifth Congress of the European Haematology Association (educational book). 2000, 110-113.

19. Galan AM, *et al.* Preparations of synthetic phospholipids promote procoagulant activity on damaged vessels: studies under flow conditions. Transfusion. 1998, 38: 1004-1010.
20. Kitaguchi T, *et al.* Characterization of liposomes carrying von Willebrand factor-binding domain of platelet glycoprotein Ibalpha: a potential substitute for platelet transfusion. Biochemical and Biophysical Research Communications. 1999, 261: 784-789.
21. Davies AR, *et al.* Interactions of platelets with Synthocytes, a novel platelet substitute. Platelets. 2002, 13: 197-205.
22. Rivera J, Vicente V. Potential and current development of platelet products. Haematologica (educational book). 1999, 84: 115-119.
23. Kresie L. Artificial blood: an update on current red cell and platelet substitutes. Baylor University Medical Center Proceedings. 2001, 14: 158-161.
24. Angles-Cano E. A spectrophotometric solid-phase fibrin-tissue plasminogen activator activity assay (SOFIA-tPA) for high-fibrin-affinity tissue plasminogen activators. Analytical Biochemistry. 1986, 153: 201-210.
25. Davies AR, *et al.* Interactions of platelets with Synthocytes, a novel platelet substitute. Platelets. 2002, 13: 197-205.
26. Tortora GJ, Grabowski SR. Principles of Anatomy and Physiology. Publisher: Harper Collins College. 1993, 567-589.

General discussion and Summary

The present research shows that 1) *in vitro* hemodilution of whole blood with polyHb resulted in decreased platelet aggregation and incomplete coagulation, and 2) the above problem with polyHb hemodilution may benefit from a blood substitute that can enhance coagulation responses. In this thesis, a novel blood substitute was presented. PolyHb-Fg was developed through the polymerization of hemoglobin and fibrinogen in a crosslinking reaction with glutaraldehyde. *In vitro* whole blood coagulation tests showed that polyHb-Fg, in a hemodiluted setting, was able to maintain similar clotting times and clot formation as undiluted whole blood. On the other hand, the standard polyHb, in a similar hemodiluted setting, was not even able to register a clotting time. Despite this, polyHb-Fg did not increase *in vitro* aggregation in a similar hemodiluted setting.

This thesis has presented promising results in whole blood coagulation tests. PolyHb-Fg should be further analyzed for *in vitro* and *in vivo* characteristics.

Appendix

Hemoglobin concentration measurement*

Hemoglobin concentration was measured using the Drabkin's method. Hemoglobin and its derivatives, except for sulfhemoglobin, are oxidized to methemoglobin by ferricyanide. Methemoglobin is subsequently converted to cyanomethemoglobin by its reaction with cyanide, contained within Drabkin's reagent. The red coloured complex that is produced has a peak absorbance at 540nm and is proportional to the hemoglobin concentration in the solution. Drabkin's reagent contains sodium bicarbonate (1g/L), potassium cyanide (0.052g/L) and potassium ferricyanide (0.13g/L). Cyanomethemoglobin standard (0.08g/dL) is supplied with the kit. Standard and reagent solution are combined to give working standards with hemoglobin equivalency (g/dL) based on a dilution sample factor of 1:251. The procedure was performed as follows. The UV spectrophotometer was zeroed with 3mL cuvettes containing reagent solution. 20μL of the sample was added to 5mL of reagent solution in a separate glass tube and thoroughly mixed. After 15 minutes, the solution was transferred into cuvettes and the absorbance was measured at 540nm. The concentration was calculated from the standard curve (Figure 14).

* Yu B. McGill University Doctoral Thesis: Polyhemoglobin-tyrosinase and artificial cells microencapsulated tyrosinase for the removal of systemic tyrosine – a potential novel therapy for melanoma. 2002, p. 160

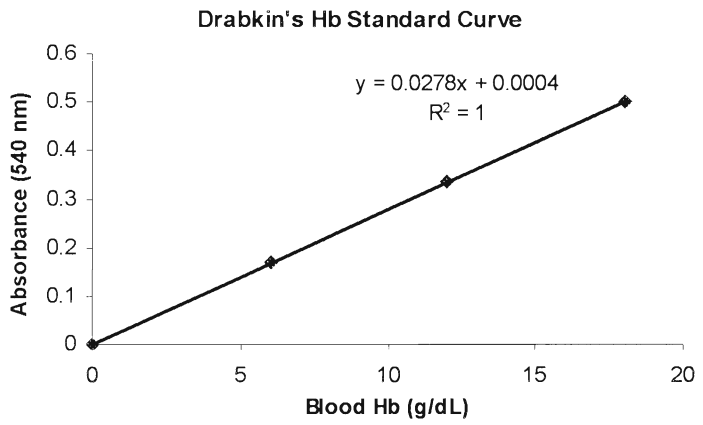

Figure 14: Hemoglobin concentration calibration curve.

Collagen concentration studies

Aggregation tests were performed with citrated whole rats blood (1:9, 0.129M sodium citrate:blood, by vol) and varying amounts of collagen for platelet activation. Five hundred microliters each of saline and whole blood was the control sample. Results are shown in Table 4.

Collagen (μL)	Average Aggregation Rate (Ω/min)
2	0.6
3.5	1.02
5	3.15

Table 4: Collagen concentration effect on aggregation rate

Other volumes of collagen were tested in different trials but there was a compromise between rates of aggregation, ease of injection (particularly with small volumes), and cost as the collagen reagent was expensive. It was decided that 3.5μL of collagen per sample

tested was the lowest volume of collagen that could be used that still gave a strong response.

Column calibration and molecular weight determination*

Sephacryl 400 HR columns used to determine molecular weights of the blood substitutes, according to the manufacturer's recommendations. The molecular weight exclusion limits were 20,000-8,000,000 Daltons for globular proteins, 10,000-2,000,000 for dextrans The void volume (V_o) of the Sephacryl 400 HR column was found to be 53.1mL determined by the initial elution peak of a sample of Blue Dextran. V_E represents the elution volume of the sample. A calibration curve was plotted as log(molecular weight x 10^3) vs V_o/V_E (Figure 15). The calibration proteins were thyroglobulin (669kD), apoferritin (443kD), β-amylase (200kD), alcohol dehydrogenase (150kD), albumin (66kD), and carbonic anhydrase (29kD).

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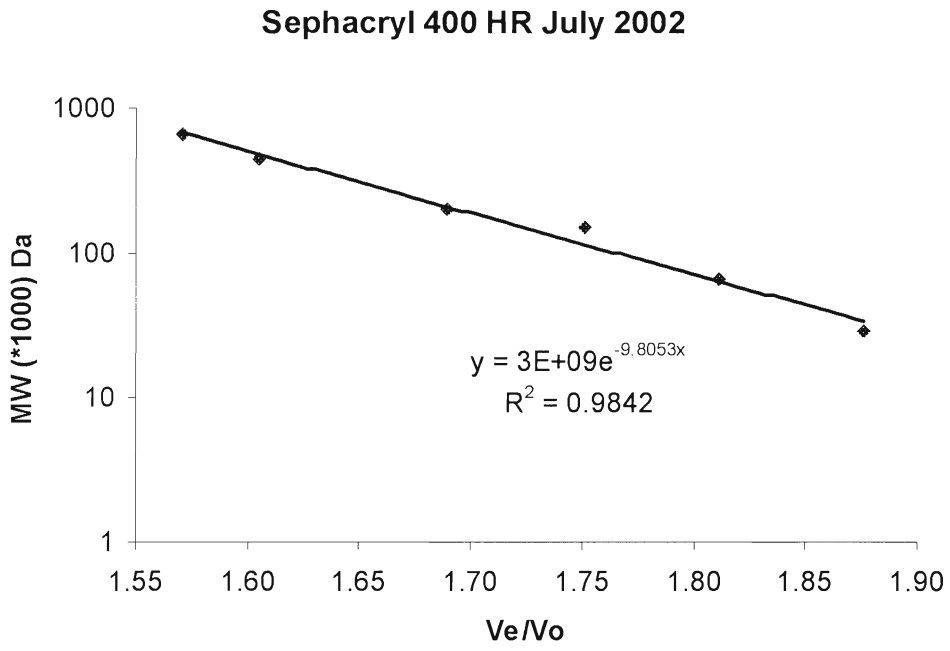

Figure 15: Gel filtration column calibration curve

Ratio screening for large polyHb and polyHb-Fg

To identify the ideal lysine and glutaraldehyde ratios for the new blood substitute, polyHb was used as a starting point. We were looking to form a blood substitute with a fairly large molecular weight that would facilitate interactions with platelets. There were a few attempts at crosslinking for 48h, but it was decided that this would decrease the oxygen carrying capabilities of the blood substitute.

With a crosslinking time at 24h, we increased the glut ratio and observed the change in molecular weights, as measured according to the gel filtration method previously described in the appendix. There was a natural limit to the molecular weight since with

higher glut ratios, the polyHb sample either gelled or became extremely viscous and could not be easily filtered. The results are shown in Table 5. The maximum glut ratio where the sample was still fluid and would not clog the gel filtration column was 27:1 with 10:1 lysine.

		ratio: #:1					
Date	**Description**	**lysine**	**glut**	**RPM**	**[Hb] g/dL**	**Fraction #**	**Description**
Aug-21	SFHb	-	-	-	9.85	33.2	symmetric peak
Aug-22	24h xlink	10	16	150	9.85	33.3	Front leading, tip at fraction 23.7
Aug-23	24h xlink	10	20	150	9.85	33.1	Front leading, tip at fraction 22
Aug-28	24h xlink	10	25	150	9.85	33.3, 18	small peak at 18, gentle fall from 33.3
Aug-28	24h xlink	10	30	150	9.85	-	gelled in less than two hours
Aug-29	24h xlink	10	28	150	9.85	33.6, 18.2	big peak at 18, reached exclusion limit, mw about 140,000,000, mucous-like viscosity
Sep-03	24h xlink	10	27	150	9.85	-	gelled before adding 4th aliquot, probably lower than 9.85 g/dL
Sep-04	24h xlink	10	28	200	9.46	-	gelled in less than one hour

Table 5: Screening for larger polyHb

With the polyHb-Fg samples, the added Fg made the samples more viscous, even with the added liquid of the Fg solution. This caused the glut ratio to decrease, depending on the amount of Fg added to the sample and in which format. Table 6 shows the trials of polyHb-Fg with different glut ratios. After the initial screening, a glut ratio of 24:1 with 10:1 lysine was used as the standard. With further trials that are described in the manuscript of this thesis, lower ratios were used to further reduce the viscosity of the sample.

		ratio: #:1					
Date	Description	lysine	glut	RPM	[Hb] g/dL	Fg concentration	Description
Sep-09	24h xlink	10	20	150	9.85	11mg in 1mL	no problems
Sep-09	24h xlink	10	25	150	9.85	11mg in 1mL	mucousy, not homogeneous
Sep-13	adsorbed Fg	10	20	150	9.85	11mg in 1mL	24hr x-link, then 4 hours adsorption
Sep-18	24h xlink	10	25	150	9.52	11mg in 1mL	mistimed, only 2 hr x-linking
Nov-11	fractionate with column	10	25	150	9.56	11mg in 1mL	gelled the first time (10.21 g/dL), redid it and ran through column to separate free Fg
Nov-17	24h xlink	10	25	150	9.85	11mg in 1mL	gelled the first time (10.36 g/dL), redid it still a bit mucousy
Dec-02	increase Fg levels	10	24	130	9.44	350mg in 5mL saline	new membrane but doesn't seem to work well
Dec-02	increase Fg levels	10	24	130	9.44	750mg in 4mL saline	

Table 6: Screening for larger polyHb-Fg

Screening tests for clotting times

Method

Clotting times were initially measured using citrated rats blood as collected for the aggregation experiments. The action of the anticoagulant was reversed by adding a volume of 0.025M $CaCl_2$ solution that would be equivalent to the calcium that is bound by the sodium citrate. The blood was maintained at 37°C and the paired polyHb/polyHb-Fg samples were citrated in a 1:9 volume ratio in order to match the conditions of the blood.

Glass tubes were placed in the 37°C water bath and 200μL of the $CaCl_2$ solution was added and warmed for one minute. The blood substitutes levels were tested at the following ratios (μL polyHb(-Fg)/μL blood) by volume: 0/500, 250/250, 400/100, and 500/0. Prewarmed blood substitute was added to the tube, followed by the addition of the balancing volume of prewarmed, citrated blood, where the timing began. The clotting

time was recorded when the tube could be completely inverted and the blood would not flow. Measurements were performed in triplicate.

Results

Table 7 summarizes the results of these experiments. Various samples were tested with interesting results. The first set was a 10:1 lys, 24:1 glut sample with 350mg of Fg in 4mL of saline added after 20h of crosslinking. The majority of the polyHb-Fg samples clotted and stuck to the sides of the tube whereas while the polyHb samples formed clots in similar time frames, none of them stuck. It should be noted that there were Fg aggregates observed in the polyHb-Fg sample even though it was previously filtered.

The other samples tested did not stick to the sides of the walls as did the first polyHb-Fg sample. Only an unpaired sample of polyHb-Fg (10:1 lys, 20:1 glut, 40mg in 4mL RL at T=4) was also able to stick to the sides of the walls.

It was explored whether the added liquid from the $CaCl_2$ caused an overdilution that prevented the clot from adhering to the walls. Higher concentrations of $CaCl_2$ solutions were prepared which would require smaller volumes to counter the action of sodium citrate. Concentrations of 0.05, 0.1, and 0.5M $CaCl_2$ solutions were tested, but there was no improvement in the adhesion of the clots to the tubes. Blood without anticoagulant clotted firmly to the sides of the tubes.

Sample	Observations
T=20; 350mg in 4mL saline; 10:1, 24:1; 8μm filtered; dialysed	sliding clot forms in polyHb faster, but polyHb-Fg (-Fg) sticks to side of tube
T=4; 40mg in 4mL saline; 10:1, 20:1; unfiltered; not dialysed	only -Fg tested, all stick to side of tube
T=0; 90mg; 10:1, 24:1; 8μm filtered; dialysed	polyHb forms sliding clot, -Fg does NOT form clot, just thickens like unsettled jello
T=20; 90mg in 4mL RL; 10:1, 24:1; unfiltered; dialysed	no significant difference
T=0; 20/40mg; 10:1, 20:1; 8μm filtered; dialysed	polyHb qualitatively seemed to form a sliding clot more readily
T=0; 40mg; 10:1, 22:1; unfiltered; not dialysed	polyHb qualitatively seemed to form a sliding clot faster
T=0; 40mg; 10:1, 23:1; unfiltered; not dialysed	polyHb qualitatively seemed to form a sliding clot faster
T=0; 40mg; 10:1, 20:1; unfiltered; not dialysed; t=37C	no significant differences, though samples very thick
T=0; 20/10/5mg; 10:1, 20:1; unfiltered; not dialysed	all -Fg samples, no significant differences

Table 7: Screening results for clotting time

Discussion

It is interesting to note that even the presence of free Fg, and presumably Fg aggregates, can cause decreased clotting times with strong adhesion. This was tested by the addition of 250μL of citrated blood to 250μL of a 10mg/mL Fg solution and 200μL of the $CaCl_2$ solution. This indicates that positive results in these clotting time experiments may not be due to polyHb-Fg but to the free Fg and Fg aggregates that remain in the polyHb-Fg solution. If this were the case, however, then all polyHb-Fg samples would show faster clotting times, which is not observed. It may be noted that the two samples that showed positive results both have the Fg added after some initial crosslinking takes place, suggesting that more free Fg remained when the polymerization reaction stops. There was another sample, however, that did not show similar results (10:1 lys, 24:1 glut, 90mg Fg in 4mL RL at T=20).

Other clotting time results

The following results follow the method indicated in the manuscript using blood with no anticoagulant. Paired polyHb and polyHb-Fg samples showed varying results in the clotting time experiments. PolyHb-Fg samples prepared with 10:1 lys, 20:1 glut with 40mg Fg added at the beginning showed no differences between the control (Table 8). Many of the samples would show partial clot formation, but would not stick to the walls of the glass tube. With a higher glut ratio (10:1 lys, 22:1 glut, 40mg Fg at T=0), the results were similar with no significant differences between polyHb and polyHb-Fg. Neither one had clots that would stick (Table 9).

* Fg aggregates in sample

		PolyHb			PolyHb-Fg		
Blood (µL)	Substitute (µL)	Clot Formation (min)	Adhesion	Comment	Clot Formation (min)	Adhesion	Comment
100	400	>20	no	not homogeneous	>20	no	not homogeneous
100	400	>20	no	not homogeneous	>20	no	nothing
100	400	>20	no	6:00 like unset jello	>20	no	nothing
250	250	>20	no	1:00 sliding clot	>20	no	1:00 sliding clot
250	250	>20	no	1:30 sliding clot	>20	no	1:00 sliding clot
250	250	>20	no	1:30 sliding clot	>20	no	2:00 unset jello
500	0				>20	no	last sample, never clots

Table 8: Clotting times for lower glut samples

		PolyHb			PolyHb-Fg		
Blood (µL)	Substitute (µL)	Clot Formation (min)	Adhesion	Comment	Clot Formation (min)	Adhesion	Comment
100	400	>20	no	5:00 sliding clot	>20	no	7:00 small floating clot
100	400	>20	no	5.95 almost stuck	>20	no	nothing
100	400	>20	no	8 almost stuck	>20	no	nothing
250	250	>20	no	3.38 sliding clot	>20	no	4.43 sliding clot
250	250	>20	no	3.67 sliding clot	>20	no	5:00 sliding clot
250	250	>20	no	3.67 sliding clot	>20	no	5:00 unset jello
500	0				3	yes	
500	0				3	yes	
500	0				3	yes	

Table 9: Clotting times for higher glut samples