



Translational feasibility of soluble nanobiotherapeutics with enhanced red blood cell functions

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Introduction

General

The first reports on biotherapeutic artificial red blood cells (RBC) were published some time ago [1,2]. Most people thought that artificial RBC was a simple matter that could be quickly developed for clinical use when needed. Thus, only the other areas of artificial cells were extensively developed around the world [3–5]. When AIDS came unexpectedly in the 1980s, there was no blood substitutes and many patients were infected with HIV contaminated donor blood. It was only then that intense research and development (R&D) on blood substitutes was belatedly carried out around the world. It was found out too late that blood substitute required the same long-term research as in any other medical research as for cancer and other diseases.

RBC

RBC have three major functions: (i) transport oxygen from the lung to the tissue, (ii) remove damaging oxygen radicals, and (iii) carry carbon dioxide (CO_2) from the tissue to the lung to be removed.

Hemoglobin-based oxygen carriers (HBOCs) with one RBC function, oxygen carrier

The initial urgency with HIV-contaminated donor RBC led researchers to concentrate on simple oxygen carriers without the other RBC functions. The most extensive clinical trials were based on polyhemoglobin (PolyHb) developed by Biopure on bovine PolyHb [6], and Northfield on human PolyHb [7]. These use the basic principle of intermolecular crosslinking of hemoglobin [1] in the form of glutaraldehyde-crosslinked hemoglobin first reported by Chang [2]. PolyHb has no blood group and can be pasteurized to inactivate HIV virus. After 20 years of intense R&D in USA, South Africa has approved it for routine use for anemia in surgery, because they still have HIV-contaminated blood problem [6]. In a 2009 US multicenter clinical trial of about 710 traffic accident patients, this has been given right in the ambulance without the need for blood group testing [7]. This can delay the need for blood transfusion for 12 h compared to 30 min for the control saline group. A 3% cardiac side effect compared to 0.6% in the control group raises discussion on risk/benefit in patients who would otherwise die if no RBC is available [7]. There have been considerable discussions regarding what causes the side effects [8].

Soluble nanobiotherapeutic consisting of hemoglobin and antioxidant enzymes

This can be a soluble complex formed by crosslinking hemoglobin (Hb) with two RBC antioxidant enzymes, superoxide dismutase (SOD), and catalase (CAT) to form Poly-[Hb-SOD-CAT] [9]. Later, conjugated hemoglobin containing synthetic antioxidants (PNPH) [10] have also been prepared. Both of these can prevent nitric oxide depletion and also prevent ischemia–reperfusion injury. Other approaches discussed in details elsewhere [8].

Soluble nanobiotherapeutic with enhancement of all three RBC functions

Do we need all three RBC functions in some conditions as in sustained severe hemorrhagic shock? Sim et al. [11] show in animal study that increase intracellular pCO_2 is related to increase fatality in severe hemorrhagic shock. They also show that intracellular pCO_2 is not the same as blood pCO_2 . Tronstad et al. [12] show that increase intracellular pCO_2 is correlated with myocardial ischemia. We, therefore, add carbonic anhydrase (CA) to form a novel soluble nanobiotechnological complex Poly-[Hb-SOD-CAT-CA] with enhanced RBC functions for oxygen transport, oxygen radical removal, and CO_2 transport [13].

Soluble nanobiotherapeutics: Poly-[Hb-SOD-CAT-CA]

We have recently crosslinked Hb, SOD, CAT, and CA into a soluble Poly-[Hb-SOD-CAT-CA] nanobiotechnological complex [13]. It not only has all three RBC functions, it can have enhancement of all three RBC functions by increasing the concentrations of RBC enzymes in the complex. Thus, we can have the same RBC enzyme activity or we can also enhance the enzyme activity of Poly-[Hb-SOD-CAT-CA] to two, four or six times that of RBC [14].

Result in a 90 min severe hemorrhagic shock rat model

We studied this in rats with 90 min hemorrhagic shock at 30 mmHg mean arterial blood pressure (MAP) by removing two-third of blood volume [13]. Poly-[Hb-SOD-CAT-CA] with enhanced enzymes was significantly ($P < .05$) superior to blood in lowering of the elevated tissue pCO_2 (Figure 1); recovery of the elevated ST (Figure 2); lower troponin levels,

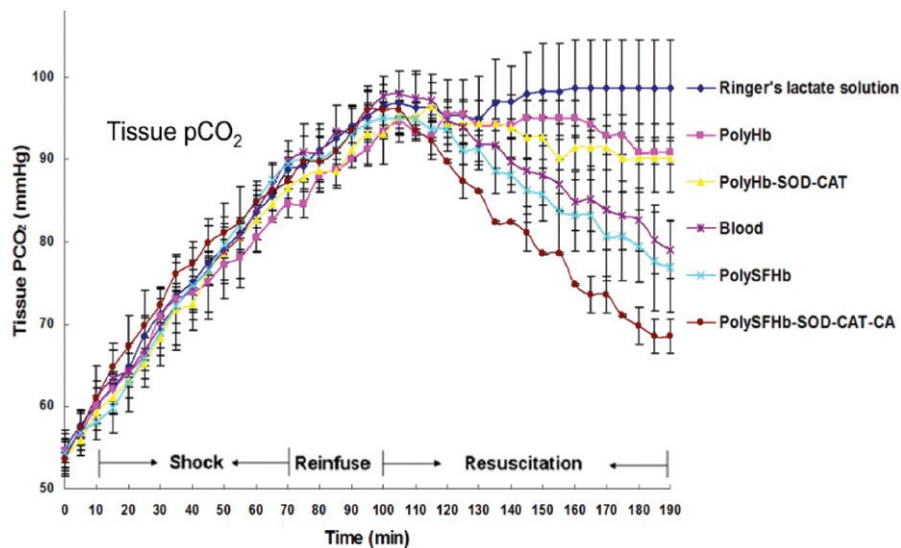


Figure 1. Effect of 90 min sustained hemorrhagic shock on intracellular pCO₂ and effects of different transfusion fluids. From Bian and Chang [13] with copyright permission.

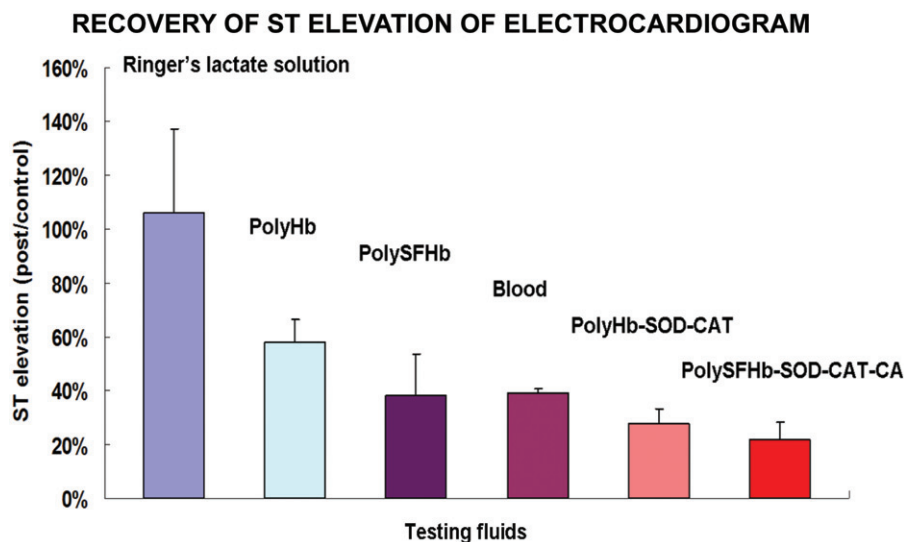


Figure 2. Effects of different transfusion fluids on the recovery of ST elevation in 90 min sustained hemorrhagic shock rat model. From Bian and Chang [13] with copyright permission.

lowering of elevated lactate, histology of the intestine, kidney, and heart [13]. PolySFHb is the crosslinking of stroma-free hemoglobin that contains hemoglobin and RBC enzymes at the concentration normally present in the RBC. It shows the same effectiveness as RBC and superior to PolyHb (Figures 1 and 2) [13].

Striking changes are observed in the histology of the intestine [13]. When reperfused with the animal's own blood, there is some detachment of the epithelium from the villi suggesting some tissue injuries but the gland architecture is still intact (Figure 3). The injury in the PolyHb group (Figure 4) shows injured villi and some damage to the glands but most of the glands still retain their structure. Poly-[Hb-SOD-CAT-CA] (Figure 5) show intact mucosal structure with no obvious injuries.

What is the translational prospect for clinical use of this nanobiotherapeutic?

The following questions have to be resolved in order for us to see if this has any prospect for translation towards clinical applications. These include storage stability, pasteurization, costs and source of enzymes, and immunogenicity.

Storage stability

Poly-[Hb-SOD-CAT-CA] contains both Hb and enzymes, and enzymes are particularly sensitive to storage and heat. We solve this problem by freeze drying this into lyophilized powder that can be easily reconstituted into solution when needed [15]. Our recent detailed analysis shows that this extends the storage time of Poly-[Hb-SOD-CAT-CA] to 1 year

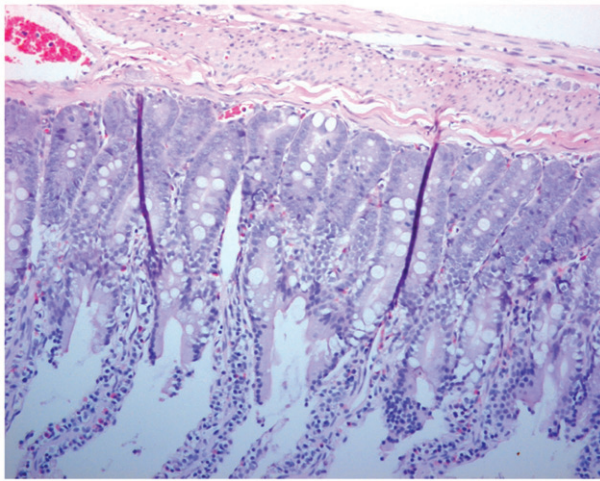


Figure 3. Hematoxylin and eosin staining of intestine tissue sections (200 \times) of Shed blood reperfusion group after 90 min ischemia and 1 h resuscitation.

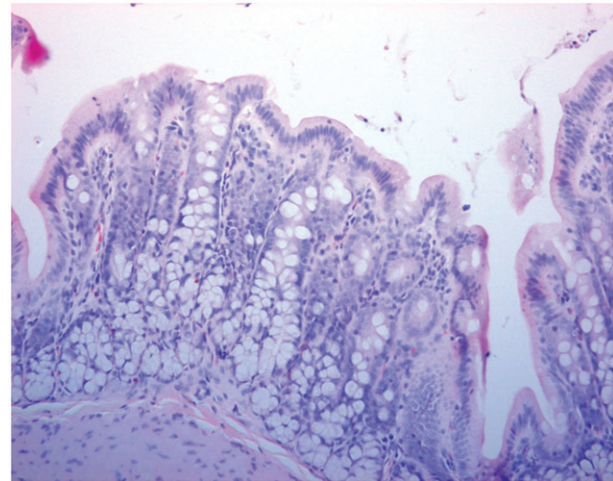


Figure 5. Hematoxylin and eosin staining of intestine tissue sections (200 \times) of Poly-[SFHb-SOD-CAT-CA] reperfusion after 90 min ischemia and 1 h resuscitation.

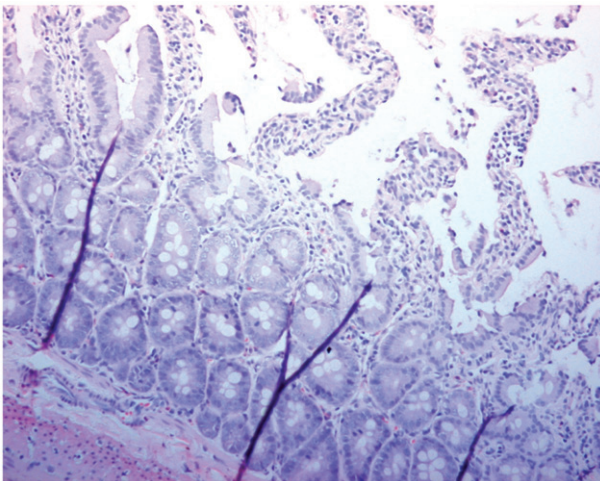


Figure 4. Hematoxylin and eosin staining of intestine tissue sections (200 \times) of PolyHb reperfusion after 90 min ischemia and 1 h resuscitation.

at 4 °C (compared to 42 days for RBC) and 40 days at room temperature (compared to 1 day for RBC) [15]. Poly-[Hb-SOD-CAT-CA] retains its oxygen carrying ability, P50 value, before and after long-term storage. After the freeze-dry process, the enzyme activities are $100 \pm 2\%$ for CA, $100 \pm 2\%$ for SOD, and $93 \pm 3.5\%$ for CAT.

Pasteurization

Unlike RBC or the solution form, the lyophilized form can be heat pasteurized at 70 °C for 2 h to retain good enzyme activities of CA $97 \pm 4\%$, SOD $100 \pm 2.5\%$, and CAT $63.8 \pm 4\%$ [15]. Adding more CAT in crosslinking can maintain the same enzyme ratio after pasteurization. Further investigation is needed to study the potential use of pasteurization of the lyophilized preparation as an additional step to the preparative procedure that involves crosslinking with glutaraldehyde and ultrafiltration that can inactivate or remove infective agents. FDA has already approved the sterilization method used for PolyHb.

Costs of enzymes

Purified enzymes from commercial sources are extremely expensive. Our research resulted in a novel method to simultaneously extract SOD, CAT, and CA from the same sample of RBC [14]. This avoids the need for expensive commercial enzymes thus allowing this to be cost-effective for future large-scale production of a nanobiotechnological Poly-[Hb-SOD-CAT-CA] with enhancement of all three RBC functions. The best concentration of phosphate buffer was analyzed and established resulting in good recovery of CAT, SOD, and CA after extraction [14]. Different concentrations of the extracted enzymes can be used to enhance the activity of Poly-[Hb-SOD-CAT-CA] to two, four or six times that of RBC. When conditions only require Poly-[Hb-SOD-CAT-CA] with the same concentration of enzymes as RBC, then the content of RBC can be easily use without the need for enzyme extraction.

Source of hemoglobin and enzymes

This novel extraction method [14] will also allow us to extract the needed enzymes from any RBC source including discarded or contaminated donor RBC, post-stem cell extracted human placental RBC (cord RBC), bovine RBC or other RBC. Other possible sources include recombinant enzymes or bio-engineered enzymes.

Effects of four weekly top loading including immunological effects

We are analyzing in details the effects of one-tenth blood volume top loading per week for 4 weeks. We follow the well-being, growth, biochemistry, histology, and also the important question of the immunological properties of this nanobiotechnological complex [16]. This work is ongoing with promising results. We are testing the immunological effects in rat by four weekly intravenously injection of one-tenth the total blood volume with the following three model system (Figure 6) [16]:

1. BovineHb–BovineEnzymes as a test in human for NonHumanHb–NonHumanEnzymes,

2. RatHb–RatEnzymes as test in human for HumanHb–HumanEnzymes, and
3. RatHb–BovineEnzymes as a test for HumanHb–NonHumanEnzymes.

Using very sensitive specific test for antibodies to bovine hemoglobin and bovine enzymes, we have obtained the following preliminary results. For BovineHb–BovineEnzymes, there is a slight increase in antibody to bovine hemoglobin but not to the nanoencapsulated bovine enzymes. RatHb–RatEnzymes as test in human for HumanHb–HumanEnzymes, there is no increase in specific antibodies and there is no change in blood pressure before and after each of the four weekly injection. This shows that the procedure for the preparation did not result in changes in immunogenicity. For the RatHb–BovineEnzymes as a test for HumanHb–NonHumanEnzymes, there is no increase in

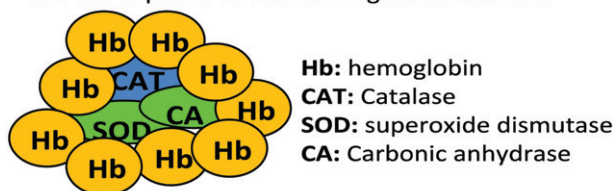
specific antibodies and there is no change in blood pressure before and after each of the four weekly injection.

It would appear that the large amount of hemoglobin nanoencapsulate the small amount of enzymes and thus separate them from immunological reactions (Figure 6). This suggests the possibility of using human hemoglobin together with nonhuman source of enzymes thereby saving the need to extract enzymes from human RBC. Human hemoglobin could come from discarded or contaminated donor RBC, post-stem cell extracted human placental RBC (cord RBC), and recombinant sources.

2.3.6 Summary

The results obtained up to now show promise towards potential translation for clinical use. However, much more detailed preclinical studies needs to be carried out. The final test will be the result of actual clinical trial in human.

Excess hemoglobin forms a protecting barrier nanoencapsulating the enzymes so the enzymes are not exposed to immunological interaction



1. BovineHb–BovineEnzymes (NonHumanHb–NonHumanEnzymes)
2. RatHb–RatEnzymes (HumanHb–HumanEnzymes)
3. RatHb–BovineEnzymes (HumanHb–NonHumanEnzymes)

Figure 6. Different type of Poly-[Hb-SOD-CAT-CA].

Discussions

Comparison of different approaches

Progress in therapy is a stepwise process and we now have more experience and knowledge in this area. We can now tailor-make blood substitutes ranging from simple oxygen carriers to complex nanobiotherapeutics (Table 1). However, if a condition only needs oxygen carriers then it would not be cost-effective to use a more complex nanobiotherapeutic. On the other hand, it would be folly not to use a more complex nanobiotherapeutic if indicated. Table 1 is a brief summary comparing PolyHb, Poly-[Hb-SOD-CAT], and Poly-[Hb-SOD-CAT-CA] with RBC.

Table 1. Comparison of PolyHb, Poly-[Hb-SOD-CAT] and Poly-[Hb-SOD-CAT-CA] with RBC.

Properties	RBC	Poly-[Hb-SOD-CAT-CA]	Poly-[Hb-SOD-CAT]	PolyHb
Oxygen transport	7 microns	Soluble: better perfusion in partial arterial obstruction	Soluble: better perfusion in partial arterial obstruction	Soluble: better perfusion in partial arterial obstruction
Oxygen radicals removal	7 microns RBC enzyme	Soluble + enhanced enzymes better perfusion and removal of oxygen radicals	Soluble + enhanced enzymes better perfusion and removal of oxygen radicals	N/A
Carbon dioxide transport	7 microns RBC Enzymes	Soluble + enhanced enzymes better perfusion and CO ₂ removal	N/A	N/A
Storage	Suspension <1 day 20 °C, 42 days 4 °C	Lyophilized 40 days 20 °C, >320 days 4 °C	Lyophilized 40 days 20 °C, >320 days 4 °C	Solution >320 days 20 °C
Blood groups	Yes unless O, needs blood group matching and typing	None Can be given on the spot	None Can be given on the spot	None Can be given on the spot
Heat pasteurization	Cannot	Yes for lyophilized form	Yes for lyophilized form	Yes
Platelet-like function	N/A	Possible if fibrinogen xlinked [17]	Possible if fibrinogen xlinked [17]	Possible if fibrinogen xlinked [17]
Availability	Limited human source	Human and nonhuman sources	Human and nonhuman sources	Human and nonhuman sources
Circulation time	Donor RBC 30 days	1–2 days	1–2 days	1–2 days
Potential areas of application	Better for elective long-term RBC replacement Especially chronic anemia and other clinical conditions	As nanobiotherapeutic for emergency uses and therapy in conditions requiring all three enhanced RBC functions (i) O ₂ transport, (ii) oxygen radical removal and (iii) CO ₂ transport	As nanobiotherapeutic for emergency uses and therapy in conditions requiring enhanced (i) O ₂ transport and (ii) oxygen radical removal	As nanobiotherapeutic for emergency uses and therapy in conditions requiring enhanced (1) O ₂ transport

N/A: not applicable.

Potential implications of poly-[Hb-SOD-CAT-CA] in medicine

Under normal circumstances, donor blood is the best replacement for blood. However:

1. Natural epidemics (e.g. HIV, Ebola, Zika, etc.) or man-made epidemics (terrorism, war, etc.) can result in contaminated donor blood or disqualified disease contact donors. Unlike RBC or the soluble form, lyophilized Poly-[Hb-SOD-CAT-CA] can be heat pasteurized in addition to ultrafiltration and glutaraldehyde crosslinking. FDA has already approved the sterilization method used for PolyHb
2. Severe blood loss from accidents, disasters, terrorism or war may require urgent blood transfusion that cannot wait for transportation to the hospital for blood group testing—especially in the frontline or remote area especially northern regions of Canada and China. Unlike RBC, Poly-[Hb-SOD-CAT-CA] does not have blood groups and can be given on the spot.
3. In very severe hemorrhagic shock there is usually a safety window of 60 min for blood replacement, beyond which there could be problems related to irreversible shock. Our animal study shows that Poly-[Hb-SOD-CAT-CA] with enhanced RBC enzymes could prolong the safety window.
4. Heart attack and stroke can be caused by obstruction of arterial blood vessels. Unlike RBC particles, this nanobio-therapeutic being a solution can more easily perfuse through partially obstructed vessels to reach the heart and brain. Furthermore, its enhanced antioxidant enzymes (SOD and CAT) can effectively remove oxygen radicals to prevent ischemia reperfusion injury to the reperfused tissues.
5. RBC have to be stored in refrigeration thus more difficult to transport and store in disaster, frontline, and remote areas. We show that lyophilized Poly-[Hb-SOD-CAT-CA] can be stored at room temperature for more than 40 days, compared to RBC of 1 day at room temperature. It can be stored in refrigeration at 4°C for more than 320 days as compared to RBC of 42 days.

Concluding remarks

In clinical medicine, there is no one single approach for all conditions. When the clinical condition only requires an oxygen carrier like PolyHb, then there is no need to use the more complex and more expensive Poly-[Hb-SOD-CAT-CA]. There are also conditions that may only require PolySFHb with the same enzyme functions as RBC. Other conditions may only require oxygen carrier with antioxidant functions. On the other hand, there are also conditions that may need Poly-[Hb-SOD-CAT-CA] with enhancement of all three RBC functions.

Translation to clinical use is very time consuming and we should learn from past experience not to wait until it is again too late. We should also analyze how research in animal studies can be applied to clinical use in patients. We also need to

continue to look into the future since biological therapy undergoes rapid progress with many other future possibilities [8,18].

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Disclosure statement

The author report no conflict of interest. The author alone are responsible for the content and writing of this article.

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