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NANOBIOTECHNOLOGY FOR HEMOGLOBIN BASED BLOOD SUBSTITUTES

Thomas Ming Swi Chang, OC,MD,CM,PhD,FRCPC,FRS[C],
Professor and Director, Artificial Cells & Organs Research Center,
Departments of Physiology, Medicine & Biomedical Engineering, Faculty of Medicine,
McGill University, 3655 Drummond Street, Montreal, Quebec, Canada H3Y1Y6.
e-mail: artcell.med@mcgill.ca website: www.artcell.mcgill.ca

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Abstract

Nanobiotechnology is the assembling of biological molecules into nanodimension complexes. This has been used for the preparation of polyhemoglobin formed by the assembling of hemoglobin molecules into soluble nanodimension complex. New generations of this approach includes the nanobiotechnological assembly of hemoglobin, catalase and superoxide dismutase into a soluble nanodimension complex. This acts both as oxygen carrier and antioxidant for those conditions with potentials for ischemia-reperfusion injuries. Another recent novel approach is the assembling of hemoglobin and fibrinogen into a soluble nanodimension polyhemoglobin-fibrinogen complex that acts as an oxygen carrier with platelet-like activity. This is potentially useful in cases of extensive blood loss requiring massive replacement using blood substitutes resulting in the need for platelet and clotting factors replacement. A further step is the preparation of nanodimension artificial red blood cells that contain hemoglobin and all the enzymes present in red blood cells.

Introduction

Nanobiotechnology is the assembling of biological molecules into 1 to 100 nanometer dimensions. These dimensions can be the diameter of nanodimension artificial cells or particles; membranes with nanodimension thickness or nanotubules with nanodimension diameter. The first nanobiotechnological approach reported in the literature is the crosslinking of Hb into ultrathin polyHb (PolyHb) membrane for artificial red blood cell membrane [1,2]. If the emulsion is made even smaller, then the whole artificial cells with its Hb can be crosslinked into PolyHb of nanodimension. Glutaraldehyde can crosslink Hb into soluble PolyHb of nanodimension [3] . New generations of this approach includes the nanobiotechnological assembly of hemoglobin, catalase and superoxide dismutase into a soluble nanodimension complex. This acts both as oxygen carrier and antioxidant for those conditions with potentials for ischemia-reperfusion injuries (4-7). Another recent novel approach is the

assembling of hemoglobin and fibrinogen into a soluble nanodimension polyhemoglobin-fibrinogen complex that act as an oxygen carrier with platelet-like activity (6). This is potentially useful in cases of extensive blood loss requiring massive replacement using blood substitutes resulting in the need for platelet and clotting factors replacement. Nanodimension artificial cells can also be formed in the form nanodimension biodegradable polymeric membrane artificial cells containing Hb and rbc enzymes[4-6]

Polyhemoglogin

Basic principles
Hb is a tetramer (α1β1α2β2) (8)that breaks down into toxic dimers (α1β1 and α2β2) that cause renal toxicity and other adverse effects. Even in the form of tetramers, Hb molecules can cross the intercellular junction of blood vessels to cause adverse vasopressor effects. We have used the principle of nanobiotechnology to assemble Hb molecules into nanodimenion polyHb first using bifunctional agent sebacyl chloride [1-2], then glutaraldehyde [3]. The glutaraldehyde method has been developed independently by other groups for clinical trials (9,10)

Present status of PolyHb in clinical trials
Gould's group has carried out Phase III clinical trials on 171 patients showing that this product can successfully replace extensive blood loss in trauma surgery by maintaining the Hb level at the 8 to 10 g/dl needed for safe surgery with no reported side effects [9]. For example, transfusion of this polyHb in patients with Hb level as low as 2g/dl can raise the Hb level to within the 8 to 10 g/dl level with the patients recovering from surgery. Normally patients with Hb levels of <3% do not survive. Gould's has infused up to 10 litres of polyHb into individual trauma surgery patients. In the USA this product has been approved for compassionate use in patients and it is waiting for regulatory decision for routine clinical uses. They have carried out Phase III clinical trials on its used in pre-hospital emergencies since no typing and cross-matching is needed and it can be used right on the spot. These clinical trials have not yet been reported in the literature but the protocol and results have been discussed on their website (www.northfieldlabs.com).
Given that the supply of Hb from outdated donor blood is limited, a glutaraldehyde-crosslinked bovine polyHb has been developed and tested in phase III clinical trials [10]. For example, they have carried out a multicenter, multinational, randomized, single-blind, RBC-controlled Phase III clinical trials in patients undergoing elective orthopedic surgery. A total of 688 patients were randomized 1:1 to receive either the polyHb or RBC at the time of the first perioperative RBC transfusion decision and 59.4% of the patients receiving polyHb required no RBC transfusion all the way to follow up and 96.3% avoided transfusion with RBC on the first postoperative day and up to 70.3% avoided RBC transfusion up to day 7 after. In North America, this polyHb has been approved for compassionate uses in patients and in South Africa, this has been approved for routine use.

Effects of Tetrameric Hb in polyHb on vasoconstriction and adverse cardiac effects

1. *In addition to the above two polyhemoglobins, other modified hemoglobins have also been prepared and studied. These include intramolecularly crosslinked tetrameric hemoglobin and also polyhemoglobin containing more than 30% tetrameric*

hemoglobin that can cause vasoconstriction. This has led to the proposal that the intercellular junctions of the endothelial lining of vascular wall allow molecular dimension Hb to enter into the interstitial space. There, Hb acts as a sink in binding and removing nitric oxide needed for maintaining the normal tone of smooth muscles. This results in the constriction of blood vessels and other smooth muscles especially those of the esophagus and the GI tract. To test this we prepare PolyHb each containing different percentage of unpolymerized Hb molecules using the same glutaraldehyde crosslinking and characterized to ensure that they all have the same oxygen affinity (11). The result shows that the one with the lowest % of unpolymerized Hb molecules does not cause vasoconstriction.With increasing % of unpolymerized Hb molecules, there was increasing degree of vasoconstriction Rat hearts have very high heart rates and therefore more sensitive to ischemic changes. Our recent studies in rats show ischemic ECG changes in the form of ST elevation when rats received polyhemoglobin with high % of tetrameric hemoglobin. With even higher % of tetrameric hemoglobin, there was ECG evidence of cardiac arrhythmia. ST elevation could be due to vasoconstriction resulting in decrease supply of oxygen to the heart and this may explain the observation of small subendocardial lesions in some primates and swine after infusion with one type of modified Hb consisting of 100% single Hb molecules. Thus, in order not to cause adverse vasopressor or cardiac effects, polyHb preparation must contain less than 2% of tetrameric Hb.

New generation of nanobiotechnology based blood substitutes: assembling Hb with catalase and superoxide dismutase.

Rationales
PolyHb is likely to have an important role in certain clinical applications. However, for conditions with potentials for ischemia reperfusion injuries we have to use a new generation of blood substitute that is both an oxygen carrier and an antioxidant. The reasons for this is that lack of oxygen supply in ischemic heart, sustained hemorrhagic shock, stroke, myocardial infarction, organ transplantation and other conditions may result in ischemia. Ischemia leads to alterations in metabolic reactions producing hypoxanthine and activating the enzyme xanthine oxidase. The level of hypoxanthine increases with the duration and severity of ischemia. When the tissue is reperfused with oxygen carrying fluid, xanthine oxidase converts oxygen and hypoxanthine into superoxide. By several mechanisms, superoxide results in the formation of oxygen radicals that can cause tissue injury.
Even in routine surgery, it will be important to rule out patients with cardiac ischemia when using modified hemoglobin with no antioxidants. Otherwise, there could be adverse cardiac effects related to ischemia-reperfusion. Other conditions where ischemia reperfusion injuries would be more likely include severe sustained hemorrhagic shock, stroke, mycarodial infarction, organ transplantation and others. For all the above situations we have used nanobiotechnology to assemble Hb with CAT and superoxide dismutase into soluble nanodimension PolyHb-CAT-SOD (Figure 1) [12] This is an oxygen carrier with the ability to remove oxygen radicals. Superoxide dismutase and catalase converts superoxide into hydrogen peroxide that is in turn converted into water and oxygen.

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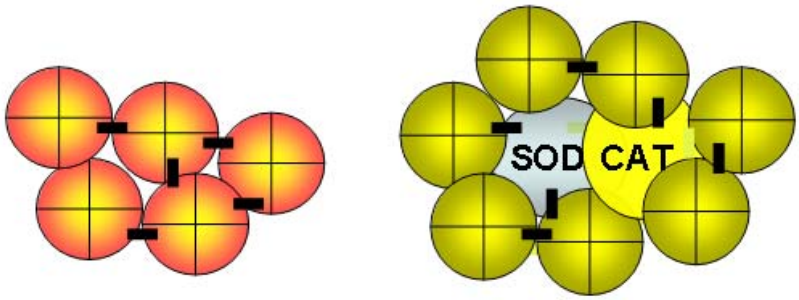

Figure 1: Left: Polyhemoglobin formed by nanobiotechnological assembling of hemoglobin molecules into soluble nanodimension complex. Right: Polyhemoglobin-catalase-superoxide dismutase formed by nanobiotechnological assembling of hemoglobin, catalase and superoxide dismutase into soluble nanodimension complex.(with copyright permission from 2007 monograph on Artificial Cells [4])

Compared to PolyHb, PolyHb-SOD- CAT removes significantly more oxygen radicals and peroxides and stabilizes the crosslinked hemoglobin resulting in decrease oxidative iron and heme release [4-6] . Crosslinking these enzymes to polyhemoglobin is important because otherwise, free SOD and catalase are removed rapidly from the circulation with a half-time of less than 30 minutes. In the form of PolyHb-SOD-catalase these enzymes circulate with a half-time more comparable to PolyHb which is about 24 hours in human. In the reperfusion of ischemic rat intestine, PolyHb-SOD-CAT significantly reduce the increase in oxygen radicals caused by PolyHb as measured by an increase in 3,4 dihydroxybenzoate [14]. We have also carried out studies on global cerebral ischemia-reperfusion in a hemorrhagic shock model[13]. This is based on bleeding anesthetized rats to hypotensive level combined with transient occlusion of both common carotid arteries. After different length of time, this was followed by the release of the occlusion of the carotid arteries and reinfusion using different types of oxygen carrying fluids. The effect on blood brain barrier was followed by Evans blue extravasation PolyHb-SOD-CAT significantly attenuated the severity of BBB disruption as compared to saline, SF-Hb, PolyHb or a solution of free hemoglobin, SOD and CAT ($P < 0.01$) [13,15]. In the same study, brain edema was followed as changes in brain water content. The changes in brain water content of PolyHb-SOD-CAT treated animals were not significantly different from that of the sham control (Figure 2). The increase in water contents of saline, SF-Hb, PolyHb and the solution of free hemoglobin, SOD and CAT were significantly higher than that of the sham control and PolyHb-SOD-CAT group by the 4th hour and increased thereafter with time ($P < 0.01$) .

The attenuation of ischemia reperfusion injuries using PolyHb-SOD-CAT shows promise for its potential role as a protective therapeutic agent in clinical situations of ischemia and oxidative stress as in stroke, myocardial infarction, sustained severe hemorrhagic shock, organ transplantation and in cardiopulmonary bypass.

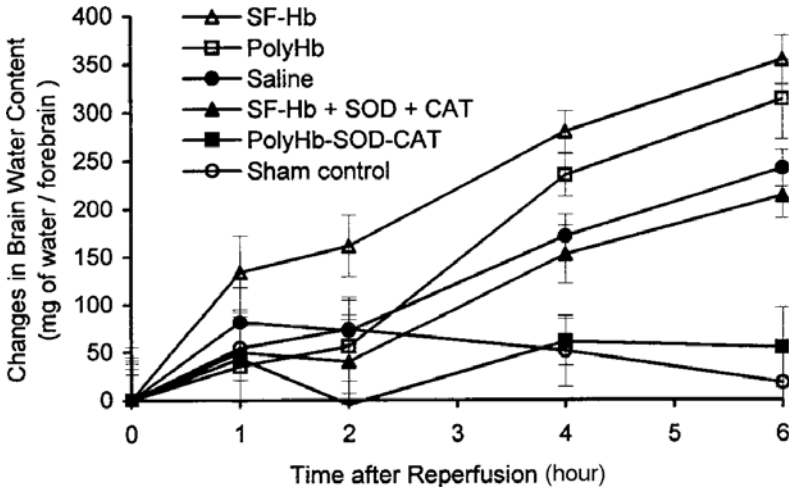

Figure 2: Brain edema: changes in brain water content. The changes in brain water content of PolyHb-SOD-CAT treated animals are not significantly different from that of the sham control. The increase in water contents of saline, SF-Hb, SF-Hb + SOD + CAT, and PolyHb are significantly different from that of the sham control and PolyHb-SOD-CAT group by the 4th h continued to increase and thereon with time. Statistical significance is $P < 0.01$. (copyright permission from 2007 monograph on Artificial Cells [4])

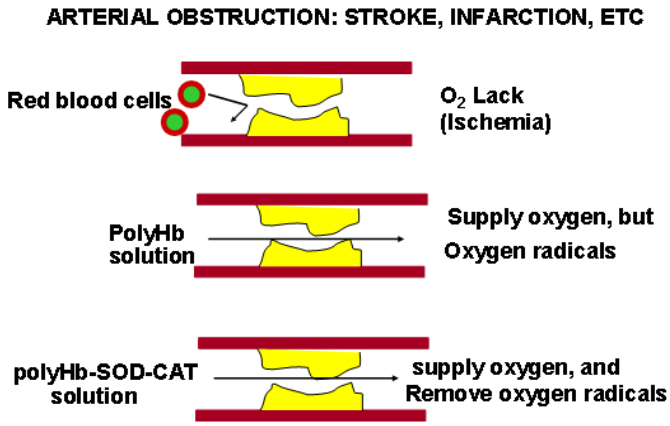

Figure 3. In obstructive ischemia polyhemoglobin supplies oxygen but may cause ischemia-reperfusion injuries. On the otherhand, polyHb-SOD-CAT can supply oxygen without causing ischemia-reperfusion injuries. (copyright permission from 2007 monograph on Artificial Cells [4])

Polyhemoglobin-Fibrinogen: a Novel Oxygen Carrier With Platelet-like Properties

In high blood volume loss, large volume red blood cell replacement alone would not replace platelets and coagulation factors and there is need to replace these also. We therefore use nanobiotechnology to develop a blood substitute that is an oxygen carrier with platelet-like properties. This is a novel blood substitute, polyhemoglobin-fibrinogen (polyHb-Fg) [16]. Briefly, PolyHb-Fg was prepared as follows. A fibrinogen solution of 40mg dissolved in 4 mL of Ringer's lactate was added 4 hours

after polymerization began. After 24 hours of polymerization, 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 dialyzed against a Ringer's lactate solution overnight.

In vitro experiments

Glass tubes were prepared with 250μL or 400μL of blood substitute. 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. 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 ($p<0.01$).

In vivo experiments

The results are shown in Figure 4. PolyHb displayed similar clotting times as polyHb-Fg for exchange transfusion of up to approximately 80%. Beyond this for PolyHb clots that formed did not always stick to the sides of the tubes and would slide freely. Beyond 93% exchange, no clots stuck for PolyHb. In contrast, the clotting times for polyHb-Fg remained normal up to 98% when there was only a slight increase in the clotting time (Figure 4).

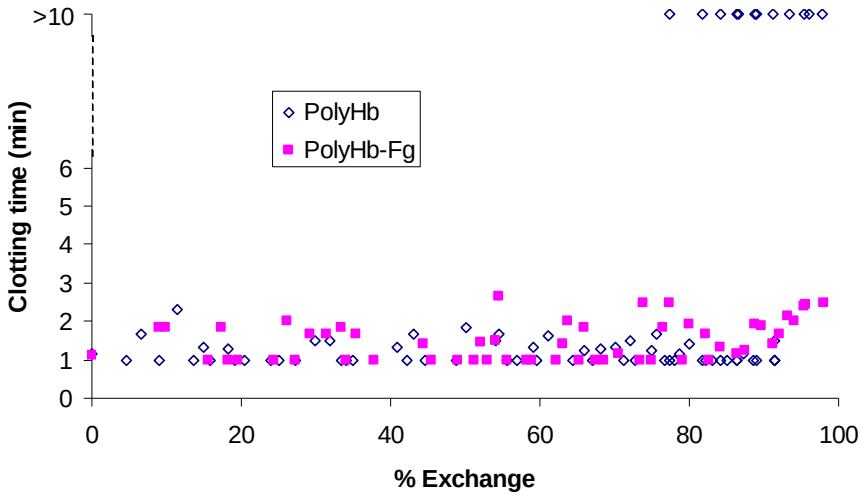

Figure 4: Exchange transfusion using polyhemoglobin-fibrinogen did not change the clotting time. However, exchange transfusion of polyhemoglobin of more than 80% resulted in clotting problems because there were insufficient platelets or clotting factors. (Copyright permission from Artificial Cells, Blood Substitutes and Biotechnoloogy [16])

Biodegradable polymeric nanodimension artificial RBCs

For this study we make use of our background in the use of biodegradable polymer like polylactide for artificial cells containing hemoglobin and other biologically active material [17]. Polylactides are degraded in the body into water and carbon dioxide. These biodegradable polymers are in routine use in surgical sutures, drug delivery and other applications. We are now using this to prepare nano-dimension

biodegradable polymer membrane hemoglobin to have mean diameter of between 80 to 200 nanometres (Figure 5)[18-21]. Polylactide is degraded in the body into lactic acid and then water and carbon dioxide. For a 500 ml suspension, the total lactic acid produced is 83 mEq [5]. This is far less than the normal resting-body lactic acid production (1000-1400 mEq/day). This is equivalent to 1% of the capacity of the body to breakdown lactic acid (7080 mEq/day).

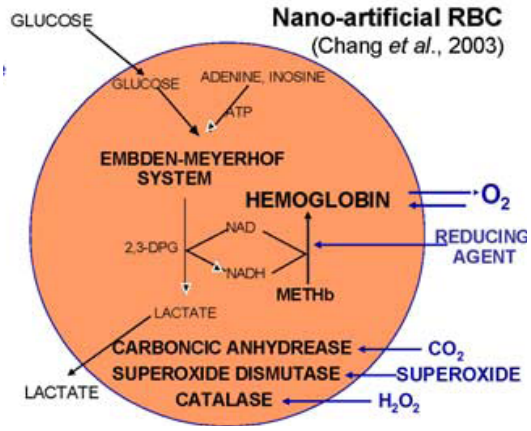

Figure 5. Nano artificial red blood cells with diameters of 80-100 nanometers containing hemoglobin and red blood cell enzymes
(Copyright permission from 2007 monograph on Artificial Cells [4])

In vitro studies
Bovine hemoglobin in these nano dimension artificial red blood cells has the same P_{50}, Bohr and Hill coefficients [4-6]. The content of hemoglobin can match that of red blood cells[4-6]. One can extract the whole content of red blood cells and then nanoencapsulate this extract. Furthermore, additional enzymes can be added to the solution before the nanoencapsulation process. Thus, additional superoxide dismutase and catalase can also be included with the hemoglobin. We also have used our background in artificial cells containing multienzyme cofactor recycling systems [23] to help solve the problem of methemoglobin formation. Applying this has helped to solve the problem related to methemoglobin formation. In nano artificial red blood cells, the biodegradable polymeric membranes can be made permeable to glucose and other molecules. This allows us to prepare hemoglobin nanocapsules containing the methemoglobin reductase system to function. External glucose can diffuse into the nanocapsules. Products of the reaction can diffuse out and therefore do not accumulation in the nanocapsules to inhibit the reaction. In vitro study shows that this can convert methemoglobin to hemoglobin[18,22]. Furthermore, reducing agents from the plasma can diffuse into the nanocapsules to reduce methemoglobin to oxygen carrying hemoglobin.[18,22]

In vivo studies
Rats have been infused with 1/3 the total blood volume. Most recently, we use a composite biodegradable polymeric membrane consisting of co-polymer of polyethylene glycol (PEG) with polylactic acid (PLA) [22]. After extensive research using this approach, we have now prepared nano-dimension artificial red blood cells that can retain their circulating hemoglobin level at double the duration of polyhemoglobin [22]. We investigate the long term effects of PEG-PLA nano artificial cells containing hemoglobin (NanoRBC) on renal and liver function and also renal,

liver and spleen histology after 1/3 blood volume top loading in rats [24,25]. The experimental rats received one of the following infusions, NanoRBC in Ringer lactate, Ringer lactate, stroma-free hemoglobin (SFHB), polyhemoglobin PolyHb), autologous rat whole blood (rat RBC). Blood samples were taken before infusions and on days 1, 7 and 21 after infusions for biochemistry analysis. Rats were sacrificed on day 21 after infusions and kidneys, liver and spleen were excised for histology examination. Infusion of SFHB induced significant decrease in renal function as shown by elevated serum urea, creatinine and uric acid throughout the 21 days. Kidney histology in SFHb infusion group revealed focal tubular necrosis and intraluminal cellular debris in the proximal tubules. In all the other groups, NanoRBC, PolyHb, Ringer lactate and rat RBC, there were no abnormalities in renal biochemistry or histology In conclusion, injection of NanoRBC did not have adverse effects on renal function nor remal histology. Nano artificial red blood cells, polyhemoglobin, Ringer lactate and rat red blood cells did not have any significant adverse effects on alanine aminotransferase, aspartate aminotransferase , alkaline phosphatase, creatine kinase, amylase and creatine kinase. On the other hand stroma-free hemoglobin induced significant adverse effect on liver as shown by elevation in alanine aminotransferase and aspartate aminotransferase throughout the 21 days. On day 21 after infusions rats were sacrificed and livers and spleens were excised for histological examination. Nano artificial red blood cells, polyhemoglobin, Ringer lactate and rat red blood cells did not cause any abnormalities in the microscopic histology of the livers and spleens. In the stroma-free hemoglobin group the livers showed accumulation of hemoglobin in central veins and sinusoids, and hepatic steatosis. In conclusion, injected nano artificial red blood cells can be efficiently metabolized and removed by the reticuloendothelial system, and do not have biochemical or histological adverse effects on the livers or the spleens. .

OTHER DIRECTIONS USING NANOBIOTECHNOLOGY

The above review only includes some examples to show the use of nanobiotechnology for the preparation of blood substitutes. This principle can be extended to other systems. One example is our study of another soluble nanodimension complex of PolyHb-tyrosinase [26,27]. This has the combined function of increasing oxygen tension to sensitize the melanoma to therapy and lowering systemic tyrosine to retard the growth of a fatal skin cancer, melanoma. Many other extensions and modifications of this general principle in nanobiotechnology are possible (27).

GENERAL DISCUSSIONS

There is always the discussions of how safe are blood substitutes. It is reasonable to require that rbc substitutes should be able to replace donor rbc without causing more adverse effect than donor rbc. One of the safety concerns regarding rbc substitutes is related to vasoactivity. As discussed above, not all hemoglobin based blood substitutes have problems related to vasoactivity and this problem is only for those that contain a high proportion of tetrameric hemoglobin. The other potential problem is related to the inappropriate use of hemoglobin based blood substitutes in those conditions that has potential for ischemia-reperfusion injuries. For these conditions, one needs to use polyhemoglobin-catalase-superoxide dismutase as discussed above. If these precautions are followed then some of the better hemoglobin based blood substitutes

could be safer that standard donar blood. After all, recent reviews show that liberal blood transfusions has a 20% increase in mortality and a 56% increase in ischemic events when compared to restrictive strategies (28,29). The transfusion of stored packed rbc is also associated with an increase in ischemic coronary events (28,30). In summary, although it is important for blood substitutes to be as safe as donor blood, it will not be reasonable to require that rbc substitutes should have no side effects while standard donor rbc are associated with adverse effects including ischemic coronary events.

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