Preprinted from:
TMS Chang (2008) Biochimica et Biophysica Acta: proteins & Proteomics 1784:1435-144

NANOBIOTECHNOLOGICAL MODIFICATION OF HEMOGLOBIN AND ENZYMES FROM THIS LABORATORY

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Abstract
Polyhemoglobin is formed by the nanobiotechnological assembling of hemoglobin molecules into soluble nanodimension complex. A further step involves the nanobiotechnological assembly of hemoglobin, catalase and superoxide dismutase into a soluble nanodimension complex. This acts both as oxygen carrier and antioxidant to prevent the oxidative effects of hemoglobin. A further step is the preparation of nanodimension artificial red blood cells that contain hemoglobin and all the enzymes present in red blood cells. Other approaches include a polyhemoglobin-fibrinogen that acts as an oxygen carrier with platelet-like activity, and a polyhemglobin-tyrosinase to retard the growth of a fatal skin cancer, melanoma.

Key words: nanobiotechnology, hemoglobin**,** polyhemoglobin, catalase, superoxide dismutase, nanomedicine, blood substitutes, fibrinogen, tyrosinase, melanoma.

Abbreviations
Hb – Hemoglobin
MetHb - metHb
PEG - polyethylene glycol.
PLA - polylactide.
PolyHb - polyHb.
RBC – red blood cells.
CAT – catalase
SOD – superoxide dismutase
RES – Reticuloendothelial system

Introduction

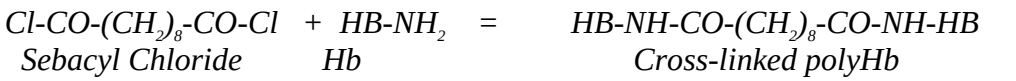
Nanotechnology is a collective term that refers to technological developments on the nanometer scale, usually 0.1-100 nm. This covers many areas including nanobiotechnology. Initially this refers to bionanoparticles. However, since many biological molecules like proteins are of nanoparticle dimensions, more detailed

definitions are ued by workers in this area. This includes the definition that 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. There are different approaches for the nanobiotechnological assembling of proteins. The first nanobiotechnological approach reported in the literature is the crosslinking of Hb into ultrathin polyHb (PolyHb) membrane of nanodimension thickness for artificial 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] . Nanodimension artificial cells containing Hb can also be formed in the form of lipid-vesicles containing Hb or nanodimension biodegradable polymeric membrane artificial cells containing Hb [4,5]

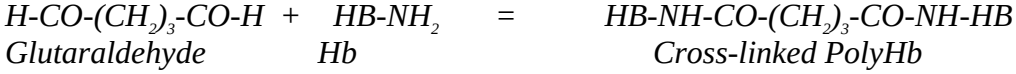
Crosslinking of Hb into PolyHb

Basic principles

Hb is a tetramer with two α subunits and two β subunits(α1β1α2β2) [6]. When Hb is infused into the body, the tetramer (α1β1α2β2) 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 [1-3]. For example, bifunctional agents can cross-link the reactive amino groups of Hb to assemble Hb molecules into polyHb (polyHb). Sebacyl chloride was the first bifunctional agent used to form membrane of nanodimension thickness [1,2].



Another bifunctional agent, glutaraldehyde was the first to be used to cross-link Hb and trace amount of CAT into soluble nanodimension structure [3].



In the glutaraldehyde procedure, cross-linking is adjusted so that the crosslinked PolyHb is in a soluble state. This cross-links protein molecules to one another (intermolecular). It can also cross-link the protein internally (intramolecular)

Tetrameric Hb in polyHb

In order not to cause adverse vasopressor effects, polyHb preparation must contain less than 2% of tetrameric Hb. Otherwise, infusion can result in vasopressor effects as the tetramer crosses the intercellular junctions of blood vessels to bind nitric oxide needed for normal vasoactivity. This cannot be done just by increasing the degree of polymerization alone since this would result in very large polyHb that are not stable in solution. The solution is to use suitable polymerization condition to prepare polyHb containing about 10% of tetramers. The tetramers are then removed by size exclusion chromatography and filtration using plasmaphresis membranes [4].

Immunological properties of polyHb

Even though Hb is only weakly antigenic, one has to make sure that the crosslinking of Hb into polyHb does not enhance its immunogenicity. In order to test this we analyze the in-vitro and in-vivo immunogenicity of cross-linking Hb into polyHb [4]. Repeated subcutaneous injections of rat Hb in Freud's adjuvant into rats did not increase the antibody titers. Even in the polymerized form (PolyHb) there is no increase in antibody titers using the very severe test of Freud's adjuvant. Infusion of polyHb from the same species either for the first time or under the most severe immunization condition, did not result in any adverse response. On the other hand, Hb from another species (heterologous) is slightly antigenic resulting in a very small but significant increase in antibody titre. Cross-linking Hb from another species into PolyHb results an increase in anitgenicity. However, even heterologous polyHb showed much lower antigenicity when compared with albumin. Infusion of polyHb from a different species for the first time also did not result in any adverse response. However, when heterologous polyHb is infused after the most severe immunization procedures, there are immmunological reactions. Thus, the result seems to show that polyHb from the same species would not have any immunological problems. It also shows that polyHb from a difference species also did not produce adverse effect when infused for the first time. This finding is supported by another group in actual clinical trials using large volumes of bovine PolyHb showing no adverse clinical immunology effects in human [7] What one cannot conclude from these results is how often can polyHb from another species be infused before there is immunological problem

Complement activation of human plasma as an in-vitro screening test

Response in animals is not always the same as for human. This is especially in tests for hypersensitivity, complement activation and immunology. We have devised a simple in-vitro test tube screening tests to bridge the gap between animal safety studies and use in human [4]. This consists of adding 0.1 ml of modified Hb to a test tube containing 0.4 ml of human plasma or blood. Then analyze the plasma for complement activation of C3 to C3a after incubating for one hour. The use of human plasma or blood gives the closest response to human next to actual injection. If we want to be more specific, we can use the plasma of the same patient who is to receive the blood substitute.

Fig. 1 summarizes those factors that initiate alternate and classical pathways leading to complement activation of C3 to C3a. Modified Hb may be contaminated with trace amount of blood group antigen that can form antigen-antibody complex that can activate C3 [4]. Others include endotoxin, microorganism, insoluble immune-complexes, chemicals, polymers, organic solvents and others [4]. In the preparation or production of blood substitute, different chemicals, reagents and organic solvents are used. This includes cross-linkers, lipids, solvents, chemicals, polymers and other materials. Some of these can potentially result in complement activation and other reactions in humans. Other potential sources of problems that can be screened include trace contaminants from ultrafilters, dialysers and chromatography [4]

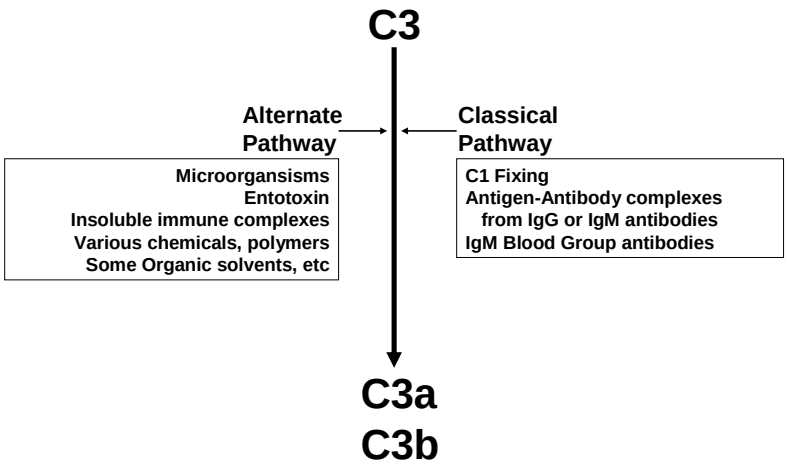


Fig. 1 Factors that activate complement in the classical and alternate pathway as shown (From[4] with copyright permission)

Present status of PolyHb in clinical trials

Gould's group has carried out Phase III clinical trials on 171 patients show that this product can successfully compensate for 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 [7]. 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.

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 [8]. 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.

Biotechnology of assembling Hb with catalase and superoxide dismutase.

Rationales

PolyHb is likely to have an important role in certain clinical applications. However, for a conditions with potentials for ischemia reperfusion injuries we have to take into consideration the issue of oxygen radicals [4,9,10]. Our aim is therefore to use nanobiotechnology to assemble Hb with CAT and superoxide dismutase into

nanodimension PolyHb-CAT-SOD that combines oxygen carrying function with the ability to remove oxygen radicals. Human superoxide dismutase and catalase are available in the recombinant form and safe for use in human. However, hterologous source of these enzymes may result in immunological reactions.

Method of preparation

Typically, each gram of Hb is crosslinked with 3000 U of SOD and 300,000 U of CAT [4,9]. The ratio of Hb to SOD and CAT (in mg/ml) is 1 : 0.009 : 0.0045. This amount of enzymes results in minimal change in the relative amount of Hb. Thus, there is minimal change in the oxygen carrying capacity of the polyHb-CAT-SOD when compared to polyHb. The ratio of enzyme can be varied over a very wide range, thus much higher amount of enzymes can be included when needed. [4,9]. During the cross-linking process, there is minimal formation of metHb when cross-linking Hb with SOD and CAT [4]. However, when cross-linking is carried out with Hb alone, there is marked formation of metHb during the preparative procedure. This shows that crosslinking Hb with SOD and CAT may also provide oxidative protection during the preparation of polyHb.

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Molecular characterization of PolyHb-CAT-SOD

Only trace amounts of antioxidant enzymes are used in relation to Hb. The ratio of Hb to SOD and CAT (as mg/ml) is typically 1:0.009:0.0045. Only 5% of the enzyme is not crosslinked into the PolyHb-CAT-SOD [11] (Table I). There is no CAT or SOD enzyme activity in the PolyHb prepared from ultrapure Hb. Crosslinking CAT, SOD and Hb with a glutaraldehyde: Hb molar ratio of 8:1 retains 90% of SOD activity and 99% of CAT activity. A higher molar ratio of 16:1 leads to a slightly lower enzyme activity of 95%.

Table I

Molecular Wt.	MW component (%)	SOD activity (%)	Catalase activity (%)
PolyHb-SOD-CAT sample			
>450 kDa	77	83 ± 3	82 ± 2
100 to 450 kDa	16	7 ± 2	11 ± 1
<100 kDa	7	4 ± 1	5 ± 1
PolyHb sample			
>450 kDa	77	0	0
100 to 450 kDa	16	0	0
<100 kDa	7	0	0

Oxygen dissociation curve

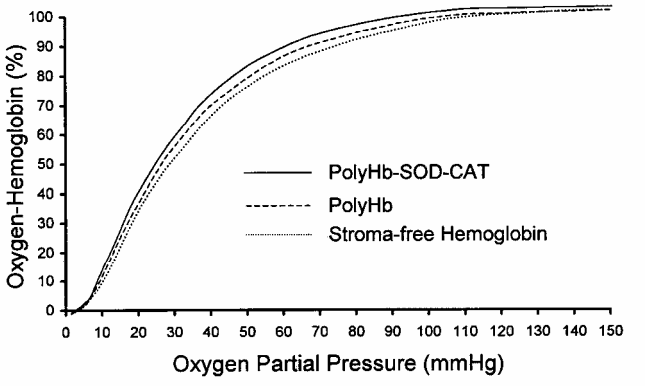


Figure 2 summarizes the results of the oxygen dissociation analysis for the 24-hours cross-linked polyHb (n=6), the 24-hours cross-linked polyHb-SOD-CAT (n=6) and the ultrapure Hb (n=6). The P_{50} for polyHb is 26.1 ± 0.2 mmHg, while that of polyHb-SOD-CAT is 24.7 ± 0.2 mmHg and ultrapure Hb is 27.8 ± 0.0 mmHg. All oxygen-Hb dissociation curves are presented as averages of 6 trials (n=6). (From [11] with copyright permission)

Effects of oxidative challenge on the degradation of Hb in PolyHb and PolyHb-CAT-SOD:

Oxygen radicals can oxidize the Fe^{2+}-heme component of Hb to Fe^{3+}-heme that can stimulate the production of superoxide ($O_2^{\bullet-}$).[9] Furthermore, excessive oxidative damage to Hb leads to the release of iron from heme. In the presence of free iron, $O_2^{\bullet-}$ and H_2O_2 rapidly react to produce hydroxyl radical ($^{\bullet}OH$) (ie. Fenton reaction).

$O_2^{\bullet-} + Fe^{3+} \rightarrow Fe^{2+} + O_2$

$H_2O_2 + Fe^{2+} \rightarrow Fe^{3+} + OH^- + {}^{\bullet}OH$

$O_2^{\bullet-} + H_2O_2 \rightarrow OH^- + {}^{\bullet}OH + O_2$

Hydrogen peroxide can also react with Hb to produce ferrylHb. These can promote cellular injury by reacting with carbohydrates, nucleic acids, and proteins. These reactive species can also readily abstract methylene hydrogen from polyunsaturated fatty acids resulting in lipid peroxidation. We carried out the following studies to see if PolyHb-CAT-SOD can prevent these effects on PolyHb.

Oxidative effects on methemoglobin formation.

We use absorbance spectra to follow the effects of superoxide and hydrogen peroxide on the Hb components of PolyHb and PolyHb-CAT-SOD [9]. For PolyHb increase in metHb (Fe^{3+}) is shown as a stepwise increase at the absorbance peak of 630 nm. PolyHb-CAT-SOD with a SOD/CAT ratio of 0.01 prevents this oxidation, while a SOD/CAT ratio of 100 produces no protective effect. In a typical study (Fig. 3), hydrogen peroxide is added to PolyHb (10 mM) or PolyHb-SOD-CAT (10 mM), and the absorbance spectra (450-700 nm) followed. For PolyHb, the spectral changes reflect the oxidation of ferrous (Fe^{2+})-heme to ferric (Fe^{3+})-heme (Fig.3 AB). The rate of degradation increases with increases in hydrogen peroxide added in amounts from 10 to 100 to 500 μM. The absorbance spectra of PolyHb-CAT-SOD are minimally affected, indicating these reactions are minimized due to the elimination of H_2O_2 (Fig 3 CD). Similar results are observed following oxidative challenge with exogenous superoxide (O_2-) produced from xanthine/xanthine oxidase.

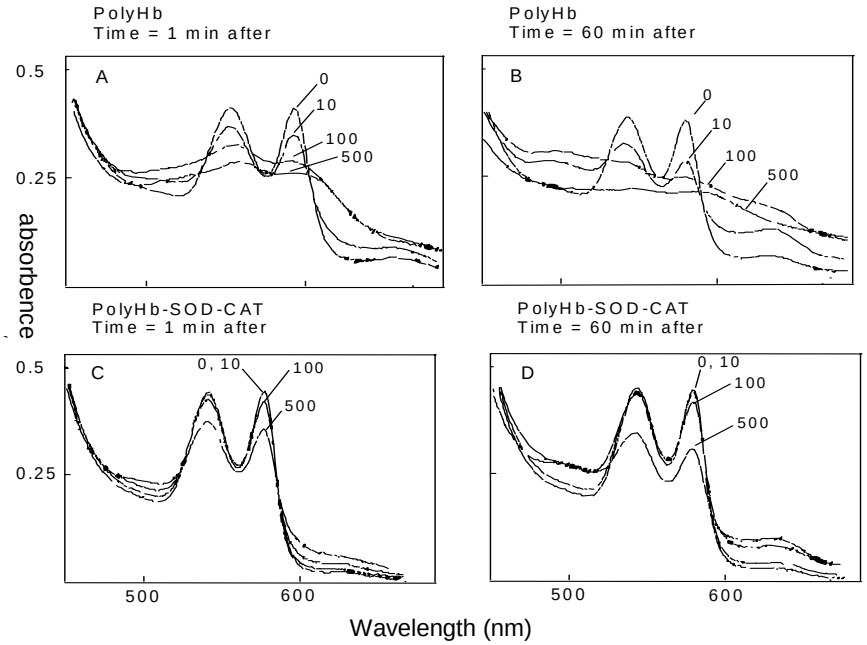


Figure 3 Absorbance spectra of PolyHb and PolyHb-SOD-CAT (10 μM) following H_2O_2 addition of 0, 10, 100, and 500 μM. (From [12] with copyright permission)

The oxidative release of iron:

The oxidative challenges on iron release from PolyHb and PolyHb-CAT-SOD are analyzed. PolyHb (15 μM) or PolyHb-SOD-CAT (15 μM) are incubated with different concentrations of H_2O_2 for 1hr at 37 °C. We use the Ferrozine assay method [13] to measure release of iron. For PolyHb the addition of H_2O_2 leads to the release of up to 37% of the total iron in PolyHb (Fig. 4). On the other hand, for PolyHb-CAT-SOD less than 1% is released over the same H_2O_2 (Fig. 4). . It is important to note that decreasing the amount of CAT crosslinked to the PolyHb-CAT-SOD decreases the protective effects on the release of iron during H_2O_2 incubation.

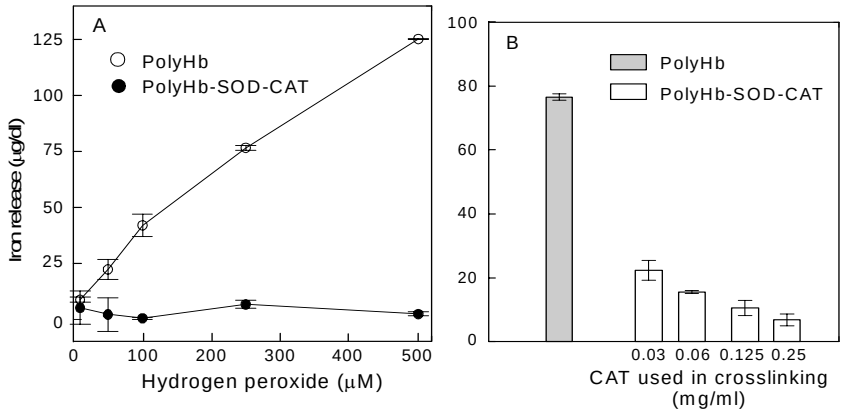


Figure 4 (A) The release of iron, measured by ferrozine assay, from PolyHb (15 μM) or PolyHb-SOD-CAT (15

μM) incubated with H_2O_2 for 1hr. at 37 °C. (B) Iron release from either PolyHb (15 μM) or PolyHb-SOD-CAT (15 μM) prepared with different CAT concentrations, following incubation with 250 μM H_2O_2. Error bars represent standard error of triplicate measurements.(From [12] with copyright permission)

Lipid peroxidation and salicylate hydroxylation

Adding 50μM or 500μM of hydrogen peroxide to PolyHb in the presence of lipid resulted in very significant increases in lipid peroxidation as shown by the production of malondiadehyde . On the other hand, the addition of 500μM of hydrogen peroxide to PolyHb-SOD-CAT causes significantly less lipid peroxidation [9] Similarly, the addition of 500μM of hydrogen peroxide to PolyHb causes salicylate hydroxylation as shown by the product 2,5 dihydroxybenzoate. However, this does not happen for PolyHb-SOD-CAT.

Ischemia reperfusion injury

In prolonged and severe hemorrhagic shock, stroke, myocardiac infarction, donor organs for transplantation and other conditions there is insufficient supply of oxygen resulting in ischemia. Ischemia stimulates the production of hypoxanthine. When the tissue is again perfused with oxygen, reperfusion, hypoxanthine is converted into superoxide. By several mechanisms, superoxide results in the formation of oxygen radicals that can cause tissue injury. Our animal studies show that PolyHb-CAT-SOD , unlike PolyHb, does not cause the release of oxygen radicals or ischemia reperfusion injury in conditions of ischemia reperfusion. Detailed results of these studies are available elsewhere [4,11]. Briefly, in the form of PolyHb-CAT-SOD the enzymes stay in the circulation with a half time of 24 hours. This is unlike the rapid removal of free SOD and CAT from the circulation (10 and 20 min., respectively). Furthermore, unless cross-linked to Hb, these enzymes are not located in close proximity to Hb, and thus are less likely to give adequate protection to Hb initiated oxygen radicals. The attenuation in hydroxyl radical generation with 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.

Artificial RBCs

The first artificial RBCs prepared in 1957 by microencapsulation of hemolysate have oxygen dissociation curve similar to RBCs [14]. We continued to study a number of microcapsule membranes including cellulose, silicone rubber, 1,6-hexamethylenediamine, cross-linked protein, phospholipid-cholesterol complexes on cross-linked protein membrane or polymer [1,2]. However, these artificial RBCs, even with diameters of down to one micron, survived for a short time in the circulation after intravenous injections. Our study shows that the long circulation time of RBCs is due to the presence of neuraminic acid on the membrane [2]. This led us to study the effects of changing surface properties of the artificial RBCs [2]. This has resulted in significant increases in circulation time, but still not sufficient for clinical uses.

Hb Lipid vesicles

Lipid membrane artificial cell
We prepared larger artificial cells with lipid membrane by supporting the lipid in the form of lipid-protein membrane and lipid-polymer membrane [2]. Others later reported the preparation of submicron 0.2 micron diameters artificial RBC using lipid membrane vesicles to encapsulate Hb [15]. This increased the circulation time significantly, although the circulation time was still rather short. Many investigators have since carried out research to improve the preparation and the circulation time. The most successful approach to improve the circulation time is to incorporate polyethylene-glycol (PEG) into the lipid membrane artificial RBC resulting in a circulation half-time of more than 30 hours [16]. Another important group has for many years carried out extensive research and development and commercial development and preclinical animal studies. Their extensive studies are available in their many publications and reviews[17,18]

Biodegradable polymeric nanodimension artificial RBCs

Introduction:
We have used a biodegradable polymer, polylactic acid (PLA), for the microencapsulation of Hb, enzymes and other biologically active material since 1976 [19]. More recently, we started to prepare artificial RBCs of 100 nanometer mean diameter using PLA, PEG-PLA membrane and other biodegradable polymers [20-22].

Characteristics
A typical electron micrograph for the biodegradable polymer Hb nanocapsules prepared with d,l-PLA shows that they are spherical and homogeneous. Their diameter ranges from 40 - 120 nm with a mean diameter of 80 nm. The membrane thickness is 5 - 15 nm. We have replaced most of the 6g/dl of lipid membrane in Hb lipid vesicles with 1.6g/dl of biodegradable polymeric membrane material. This marked decrease in the lipid component would lessen effects on the RES and lessen lipid peroxidation in ischemia-reperfusion.

Properties of Hb
We can increase the Hb content in the PLA nano artificial cell suspension from 3g/dl to 15g/dl(same as whole blood).This has normal P_{50}, Hill's coefficient and Bohr coefficients. The characteristics of the preparation containing 10.97 gm/dl of Hb prepared with d,l-PLA is listed in Table II. Nanocapsules can be prepared with up to 15 gm/dl Hb concentration.

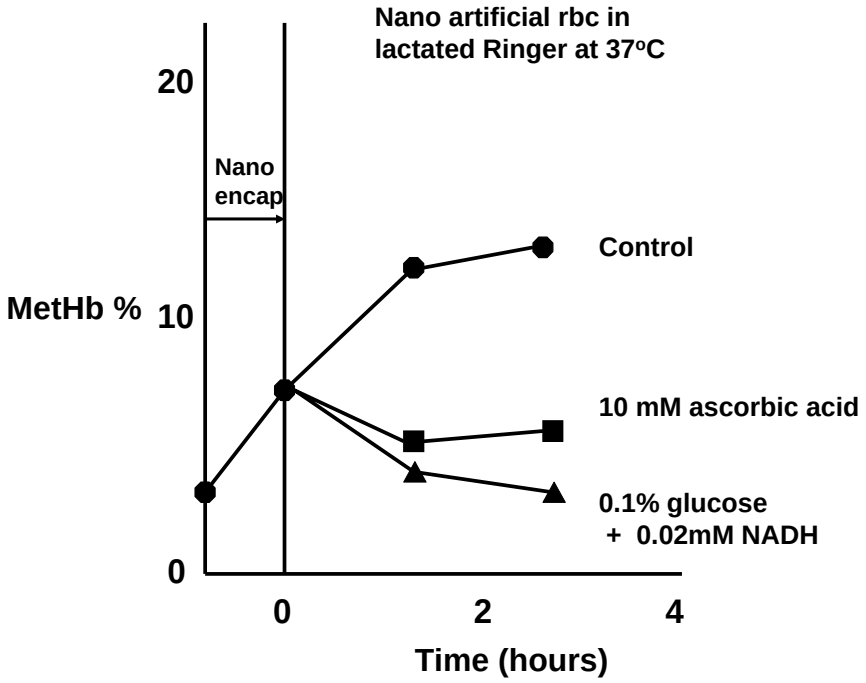
Table II. Characteristics

Hb Concentration: 10.97 g/dl (up to 15 g/dl)
Polymer concentration: 1.2 g/dl
Phospholipid concentration: 0.6 g/dl
Specific gravity (22 ^{o}C): 1.0047
Viscosity (37 ^{o}C): 3.7 to 3.8 cp
Bohr effect: -0.22 to -0.24
Hill coefficient: 2.4 to 2.9
P50: 26 mm Hg

We use the TCS Hemoxanalyser (TCS Medical Products Co., U.S.A.) to measure oxygen affinity for PLA artificial RBC with 10.97 gm/dl Hb. There is no significant difference between Hb nano artificial RBC and the original bovine Hb used for the preparation. Hill coefficient is 2.4 to 2.9. These results show that the procedure of preparation of PLA nano artificial RBCs does not have adverse effects on the Hb molecules. In the physiological pH range, oxygen affinity of PLA nano artificial RBC changed with pH ~~(Fig. 5.12)~~ and the Bohr effect is -0.22 to -0.24 [20-22].

Enzymes and multienzymes

We have earlier carried out basic research on artificial cells containing multienzyme systems with cofactor recycling [24]. A number of enzymes like carbonic anhydrase, CAT, superoxide dismutase and the metHb reductase system, normally present in RBC have been encapsulated within nano artificial RBC and retain their activities [4]. For example we have encapsulated the RBC metHb reductase system into the nano artificial RBC and showed that this can convert metHb to Hb. For PLA nano artificial RBC, the membrane can be made permeable to glucose and other small molecules. This allows us to prepare nano artificial RBC containing the metHb reductase system to function as shown in Fig. 5. Unlike lipid membrane, biodegradable polymeric membrane is permeable to glucose. Thus, the inclusion of RBC enzymes prevents MetHb formation even at 37^{o}C and we can also convert MetHb to Hb at 37^{o} C. In addition, unlike lipid membrane, the nanocapsule membrane allows plasma factors like ascorbic acid to enter the nanocapsules to prevent MetHb formation (Fig.5).



`*Figure 5 Nanoencapsulation increases MetHb level. After this when incubated at 37^{o}C metHb increases quickly. Addition of a reducing agent, ascorbic acid prevents the increase in MetHb. Addition of glucose and NADH decreases MetHb further.(From [4] with copyright permission)*

Circulation time .

In order to increase the circulation time, we synthesized a new PEG-PLA copolymer for the artificial RBC membrane [23]. After extensive research, we now have a circulation time in rats that is double that of glutaraldehye crosslinked PolyHb. Thus, After a 30% blood volume toploading using polyHb (10gm/dl), the best polyHb~~–17~~ can only attain a maximal Hb concentration of 3.35gm/dl. The best PEG-PLA artificial RBC, on the other hand, can reach a maximal Hb concentration of 3.60gm/dl. Since the RES in rat is much more efficient in removing particulate matter when compared to human, it is likely that the half time would be longer in human.

OTHER DIRECTIONS FOR POLY HB.

The above review only includes some examples to show the use of nanobiotechnology for the modification of hemoglobin and enzymes. This principle can be extended to other systems. For example, we have prepared soluble nanodimension complex of PolyHb-fibrinogen [25] that has carries oxygen and at the same time has platelet-like activities. Another example is our study of another soluble nanodimension of PolyHb-tyrosinase [26]. This has the combined function of 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. Other groups have also carried out different approaches that are not detailed in this paper that is an overview of research being carried out in this laboratory. For example, Bucci's group has developed a novel zero-link polymers of hemoglobin, which do not extravasate and do not induce pressure increases upon infusion (27). Olsen's group has synthezied a hemoglobin polymer containing antioxidant enzymes using complementary chemistry of maleimides and sulfhydryls (28)

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Acknowledgements

The author acknowledges the ongoing research grants from the Canadian Institutes of Health Research and the Quebec Ministry of Health's Hemovigillance Program in the form of a FRSQ Research Group (d'equip) on Blood Substitutes in Transfusion Medicine.