Hemoglobin Based Red Blood Cell Substitutes

Thomas Ming Swi Chang

Artificial Cells and Organs Research Center, MSSS-FRSQ Research Group on Blood Substitutes in Transfusion Medicine, McGill University, Montreal, Quebec, Canada

Abstract: Polyhemoglobin is already well into the final stages of clinical trials in human with one approved for routine clinical uses in South Africa. Conjugated hemoglobin is also in ongoing clinical trials. Meanwhile, recombinant Hb has been modified to modulate the effects of nitric oxide. Other systems contain antioxidant enzymes for those clinical applications that may have potential problems related to ischemia-reperfusion injuries. Other developments are based on hemoglobin-lipid vesicles and also the use of nanotechnology and biodegradable copolymers to prepare nano-dimension artificial red blood cells containing hemoglobin and complex enzyme systems. Key Words: Red blood cell substitutes—Artificial blood—Polyhemoglobin—Conjugated hemoglobin—Recombinant hemoglobin—Hemoglobin-lipid vesicles—Nanotechnology—Biodegradable copolymers—Oxygen therapeutics.

Serious efforts to develop modified hemoglobin (1–5) only started when native hemoglobin was shown to be toxic for human use. Research using native hemoglobin started in 1937 when Amberson prepared a hemoglobin solution obtained by lysing red blood cells for experimental transfusion in animals (6). It delivered oxygen but was highly toxic to the kidney and causes hypertension. Removal of the red blood cell membrane stroma resulted in stroma-free native hemoglobin that had less renal toxicity in the animals (7). However, Savitsky’s Phase-I clinical trial in 1978 showed that this still showed renal toxicity in addition to vasoactivities (8). The use of native hemoglobin was therefore discontinued.

MODIFIED HEMOGLOBIN

Since native hemoglobin was found to be toxic, researchers turned to Chang’s earlier basic study of encapsulated and crosslinked Hb started many years ago (9–11). Unfortunately, efforts to develop modified hemoglobin were not seriously attempted until after the disaster of HIV in donor blood. By then, it was already too late. Modified Hbs that have been developed and tested clinically include cross-linked polyhemoglobin, cross-linked tetrameric hemoglobin, and recombinant hemoglobin (2–5) (Fig. 1).

Polyhemoglobin

Bifunctional agents were first used by Chang to cross-link the reactive amino groups of Hb, producing polyhemoglobin (polyHb) (Fig. 2) (9–11). His basic research included the use of cross-linking reagents like sebacyl chloride (9,10) and glutaraldehyde (11). Glutaraldehyde-cross-linked human polyHb (11) was the basic material that has been developed independently by Gould’s group at Northfield for animal testing followed by clinical trials (12–15). Their pyridoxalated glutaraldehyde cross-linked polyHb (11) was in advance stages of Phase-III clinical trials (12,13). This preparation contains less than 1% of cross-linked tetrameric Hb (12). Their clinical trials include the use of high doses (up to 20 units or 10 L) of this preparation in trauma surgery (12,13). The group from Biopure uses glutaraldehyde cross-linked bovine polyhemoglobin (14,15). Bovine polyHb containing less than 5% cross-linked tetrameric Hb has been extensively tested in clinical trials. It has been approved for routine clinical applications in South Africa (16). Another Biopure bovine polyhemoglobin
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with slightly higher percentage of cross-linked tetramer Hb has been approved for routine use for anemia in dogs in the US. In another approach, Hsia uses a 2,3-DPG-pocket modifier prepared using dialdehyde derived from o-raffinose for forming polyhemoglobin (17,18). The group from Hemosol has been using this to form human polyHb containing 36% cross-linked tetrameric hemoglobin but clinical trial has been temporarily suspended pending further studies.

PRINCIPLES FIRST REPORTED

Crosslinked polyHb
1964 Chang – Diacid
1971 Chang – Glutaraldehyde
1980 Hsia – o-raffinose

Conjugated Hb
1964 Chang – polyamide
1968 Wong – dextran
1975 Sunder – conjugated Hb
1980 Iwashita – polyethylene glycol

Crosslinked tetrameric Hb
1968 Bunn & Jandl
1979 Walder et al. – Diaspirin

Recombinant Human Hb
1990 Hoffman et al.
1998 Doherty et al. (2nd generation)


Conjugated hemoglobin
Basic research from Chang’s group shows that Hb can be cross-linked to polymers to form an insoluble conjugated Hb (9,10). This has been extended to form soluble conjugated Hb first by Wong’s group using dextran, a soluble polymer (19). This was followed by Shorr, Iwashita, DeAngelo, and others using polyoxyethylene (20) and polyethylene glycol (PEG) (21,22). More recently, Winslow from the Sangart group has developed a new Maleimide PEG-hemoglobin that is now in Phase II clinical trial (23).

Cross-linked tetrameric Hb
Bunn and Jandl used bis(N-maleimidomethyl) ether, to crosslink Hb intramolecularly to form single cross-linked tetrameric Hb (24). The group from Baxter developed this into another cross-linker, bis(3,5-dibromosalicyl) fumarate, that cross-links the two a subunits of the Hb intramolecularly and modifies the 2,3-DPG pocket (25). However, clinical trials show that there were substantial vasoactivities and clinical studies were suspended.

Recombinant human hemoglobin
Hoffman et al. successfully prepared recombinant human Hb (26). This was developed by the group from Somatogen for clinical trials (27). Clinical trials showed substantial vasoactivities and a new recombinant hemoglobin was therefore developed to change the reactivity of Hb for nitric oxide to prevent vasoactivities (28).

Vasoactivities
We still do not know the exact reason why some of the modified hemoglobin solutions cause vasoconstriction and why there are variations between
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different types of modified hemoglobin. The most commonly accepted theory is as follows (5,12,13). The intercellular junctions of the endothelial lining of vascular wall allow single tetrameric hemoglobin to enter into the interstitial space. There, hemoglobin acts as a sink in binding and removing nitric oxide needed for maintaining the normal tone of smooth muscles. This results in vasoconstriction and the constriction of other smooth muscles especially those of the esophagus and the GI tract. The evidence for this is as follows. Gould’s group using polyhemoglobin with <1% tetrameric hemoglobin did not report vasoactivities even when large volumes of 10 units were infused. With Biopure’s bovine polyhemoglobin containing less than 5% tetrameric hemoglobin, there were only slight vasoactivities when very large volumes were used. With another type of polyhemoglobin containing 36% tetrameric hemoglobin, significant vasoactivities and increased smooth muscle contractions could be observed when using larger volumes. In the case of 100% tetrameric hemoglobin, even smaller volume would cause vasoactivities and increased smooth muscle contractions. Modified tetrameric recombinant hemoglobin that did not bind nitric oxide, also did not cause vasoconstriction (25). Furthermore, Winslow uses a conjugated hemoglobin where all the hemoglobin molecules are crosslinked to a soluble polymer, PEG. With the high water hydration of PEG, the resulting conjugated hemoglobin has a high molecular radius and thus does not cross the intercellular junction and this preparation did not cause vasoconstriction. There is another school of thought especially that from Winslow and his colleagues. Their theory is that, unlike hemoglobin inside red blood cells, modified hemoglobin being a solution is in close contact with the vascular wall. In addition they propose that modified hemoglobin in solution increases the rate of release of oxygen from red blood cells by facilitated diffusion. They propose that, the premature release of oxygen at high concentrations results in vasoconstriction. Wilson will be expanding on this in his article.

MODIFIED HEMOGLOBIN WITH ANTIOXIDANT PROPERTIES

Red blood cells contain antioxidant enzymes such as catalase and superoxide dismutase. Most of the modified-Hb blood substitutes are prepared using ultrapure hemoglobin containing no enzymes. They are effective as oxygen carriers in conditions with no prolonged ischemia as in routine surgery. However, in condition with prolonged ischemia, there is potential for ischemia reperfusion injuries (29,30). As a result modified-Hbs with antioxidant enzymes are being investigated (30–34). PolyHb-catalase-superoxide dismutase (PolyHb-SOD-CAT) is formed by cross-linking catalase, superoxide dismutase, and Hb (30–33). Compared to polyHb, PolyHb-SOD-CAT has a much lower tendency to generate free-radicals in-vitro and in-vivo (30–33). In a combined hemorrhagic shock and cerebral vascular occlusion rat model, PolyHb-SOD-CAT, unlike PolyHb, did not cause disruption of the blood–brain barrier or brain edema (33) (Fig. 3). Another group is studying the use of polynitroxylated hemoglobin with antioxidant activity (59). This chemical modification gives rise to SOD-CAT like activities.

Nanodimension encapsulated Hb

The first encapsulation of the contents of red blood cells, including Hb and enzymes, inside artificial red blood cells with artificial membranes was reported by Chang in 1957 (35). Red-blood-cell enzymes such as carbonic anhydrase (9) and catalase (35) have been included in these artificial red blood cells. The major problem was a short circulation time resulting from rapid uptake by the reticuloendothelial system (10). One of the problems was that the diameters of the microcapsules were relatively large (1–5 μm).

Hemoglobin lipid vesicles

In 1980, Djordjevich used lipid membrane liposomes to encapsulate hemoglobin with a diameter of 0.2 μm (36). These remained in the circulation for a longer time than those described above. The
groups of Rudolph and Tsuchida have carried out extensive research using lipid-encapsulated hemoglobin (37–39). Philips’ group showed that PEG–lipid vesicles are especially effective in increasing the circulation time (38). Hemoglobin lipid vesicles have been used successfully to replace most of the red blood cells in rats and also for the treatment of massive hemorrhage (37–39). Tsuchida and his collaborators are completing detailed safety and efficacy preclinical studies in animals with promising results (39).

Biodegradable-copolymer membrane nanocapsules containing Hb and red blood cell enzymes

Polylactide and polyglycolide are biodegraded in the body into water and carbon dioxide. Chang has earlier used a biodegradable polylactide polymer to encapsulate Hb and enzymes in the micron range (40) and more recently, in the nanodimension, 0.08–0.180 μm in diameter (41–43) (Fig. 4). They were able to coencapsulate superoxide dismutase catalase and methemoglobin reductase with the Hb (41,42) (Fig. 5). These nanocapsules are permeable to biological or synthetic membranes.
glucose and other small hydrophilic molecules. This makes possible the preparation of Hb nanocapsules containing the methemoglobin reductase system (42,43). Reducing agents can also diffuse into the nanocapsules to convert metHb to Hb as has been demonstrated by in vitro studies (42–45). We have recently synthesized a number of new polylactide–polyethylene glycol copolymers (PEG–PLA). One of these when used for the membrane of nanocapsules can increase the circulation time of the nanodimension artificial red blood cells to double that of polymerized hemoglobin (44–46). Infusion of one third the blood volume into rats did not result in vasoactivities. Long-term follow up has been completed and the results show that these PEG–PLA nanocapsules containing hemoglobin did not result in any changes in biochemistry, enzymes, or histology.

Oxygen therapies

In addition to their use to support the oxygen-carrying function of red blood cell substitutes, there are other potential clinical applications. As discussed earlier, polymerized hemoglobin-catalase-superoxide dismutase has been used in a rat stroke model to supply the needed oxygen without causing ischemia-reperfusion injuries (33). Another example is that tumors are not well perfused by blood but radiation therapy would be more effective with better supply of oxygen. Thus animal studies show that polymerized hemoglobin being a solution can better perfuse tumors for radiation therapy (14). More recently, we developed a polyhemoglobin–tyrosinase complex that can supply oxygen for radiation therapy and at the same time markedly decrease systemic tyrosine (47). In a mouse model, intravenous injections significantly decrease the growth of melanoma since melanoma requires tyrosine for growth (47).

DISCUSSION

In summary, Polymerized hemoglobin is already well into the final stages of clinical trials in humans. One of these has been approved for routine clinical uses in South Africa. Meanwhile, new generations of modified Hb are being developed that can modulate the effects of nitric oxide. Other systems are also being developed to include antioxidant properties for those clinical applications that may have potential problems related to oxygen radicals. A further development is the use of lipids or bio-degradable polymer membranes to prepare artificial red blood cells containing Hb and complex enzyme systems.

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