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Blood Substitutes based on Nanobiotechnology

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Stimulated by concerns of potential infective agents in donated blood, since the 1900s, there have been considerable commercial attempts to develop blood substitutes. After many years of development a few of the many leads have shown promise. This review article describes promising nanobiotechnological approaches that are now in Phase III clinical trials. This is followed by a discussion of how important basic knowledge gained is now being used to develop new generations of blood substitutes based on nanobiotechnology.

INTRODUCTION:

Since red blood cell membrane contains blood group antigens, typing and matching are needed before they can be transfused into patients. This results in delays in emergency situations. The storage time using standard method is only about 42 days. Red blood cells cannot be sterilized to remove infective agents like hepatitis viruses, H.I.V. and other potential emerging infective agents. Thus, red blood cells substitutes are being developed. Red blood cell (rbc) contains Hb, antioxidant enzymes and multienzyme system to prevent the conversion of Hb into nonfunctioning metHb. First generation rbc substitute is just an oxygen carrier based on the use of modified Hb without the presence of rbc enzymes or rbc membrane. Second generation rbc substitute is more than an oxygen carrier, it is the use of modified Hb containing rbc antioxidant enzymes. Third generation rbc blood substitute is closer to rbc, as it contains Hb and all the enzymes of rbc and the rbc membrane is replaced with synthetic membrane.

FIRST GENERATION BLOOD SUBSTITUTES

Hb (Hb) is a tetramer with two α subunits and two β subunits($\alpha1\beta1\alpha2\beta2$)[1]. Although an excellent oxygen carrier, Hb extracted from the blood cells cannot be used for infusion because it is highly toxic to the kidney[2]. Even highly purified stroma-free Hb (SFHb in which the rbc membrane stroma is removed) still showed toxicity to the kidney[3] It is now known that when free Hb is infused into the body, the tetramer ($\alpha1\beta1\alpha2\beta2$) breaks down into toxic dimers ($\alpha1\beta1$ and $\alpha2\beta2$) that causes renal toxicity and other adverse effects. The challenge is how to make use of the excellent oxygen carrying properties of Hb but prevent it from having toxic effects. There were originally 4 general methods of hemoglobin modification (Table I). Of these, only

nanotechnology based PolyHb and conjugated Hb continue to show promise in clinical trials. Intramolecularly crosslined single hemoglobin molecule and recombinant human Hb have shown adverse effects that, as will discussed later, may be related to the removal of nitric oxide.

First-generation modified Hb based on nanobiotechnology

Nanobiotechnology is the assembling of biological molecules into nanodimension structures. Hb contains reactive amino groups with many of the lysine groups being on the surface of the molecule[1]. The author was the first to use bifunctional agents to cross-link the reactive amino groups of Hb to assemble Hb molecules together into polyHb (polyHb) (www.artcell.mcgill.ca) (Fig. 1)[2,3] The first bifunctional agent used was sebacyl chloride[2]. The second bifunctional agent used was glutaraldehyde[3] Glutaraldehyde crosslinked polyHb is the basic principle that has been later developed independently by two groups with ongoing Phase III clinical trials and one of these has been approved for routine use in South Africa. One is pyridoxalated glutaraldehyde human polyHb containing <1% unpolymerized molecular Hb (www.northfieldlabs.com)[4-5].

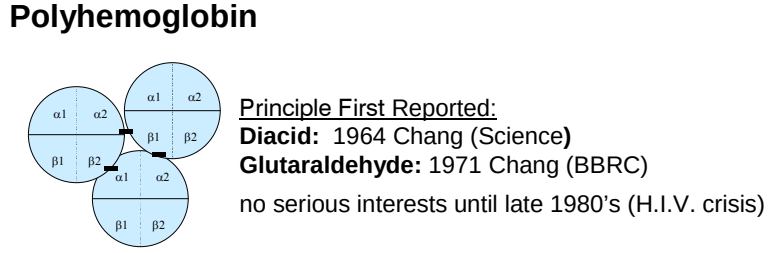


***Figure** 1 Nanobiotechnology based 1st generation Polyhemoglobin (PolyHb) (left) is formed by the intermolecular crosslinking of Hb molecules into a soluble complex containing a number of Hb molecules. In this form, they are retained in the circulation and do not cross the endothelial intercellular junction to remove nitric oxide. From Chang, TMS, (2004) Artif Cells, Blood Substitutes & Biotechnology, an international journal, 32:1- 23 Courtesy of Marcel Dekker Inc*

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[5]. 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. This group has infused up to 10 litres of polyHb into individual trauma surgery patients. They are now carrying out further 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 trails have not yet been completed but the protocol and preliminary results have been discussed on the website (www.northfieldlabs.com). 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 with <4% unpolymerized molecular Hb, has been developed and tested in phase III clinical trials (www.biopure.com)[6,7]. For example, they have carried out a multicenter, multinational, randomized, single-blind, rbc-controlled Phase III clinical trials in patients undergoing elective orthopedic surgery[6] . 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. This bovine polyHb has been approved for routine clinical use in patients in South Africa, a region with higher incidence of human immunodeficiency virus[8]. In North America, this polyHb has been approved for compassionate uses in patients.

Another way to solve the problem of supply of Hb is the recent use of red blood cells from placentas that are discarded after birth as the source of human Hb for preparing glutaraldehyde human polyHb[9] Recombinant human Hb produced by genetically engineered Escherichia coli[10,11] is another source for forming nanodimension polyHb. Other types of recombinant Hb[12,13] can also be potential sources of Hb for use in polyHb.

Why nanobiotechnology based polyHb and conjugated Hb rather than modified Hb molecules?

Modified Hb (Table I) have been successful in clinical trials[14-21]. Intramolecularly crosslinked Hb[22,23] and recombinant Hb [10] blood substitutes contain 100% of single Hb molecules. Infusion can cause vasopressor effects and also increased smooth muscle contractions. With another type of polyHb that contain 36% single Hb molecules, significant vasoactivity and increased smooth muscle contractions could also be observed when using larger volumes. On the other hand, the use of nanobiotechnology based polyHb with <1% molecular dimension modified Hb did not show vasopressor effects even when large volumes of 10 litres were infused[4-5].

Table II : Types of first generation blood substitutes

Type	Method of preparation	Notes
PolyHb based on nanobiotechnology	Glutaraldehyde is the bifunctional agent being used to crosslink Hb intermolecularly to form soluble polyHb each averaging from 3 to 10 Hb molecules.	PolyHbs with unpolymerized Hb removed do cross the intercellular junction of the endothelial cell lining of blood vessels to remove nitric oxide. As a result it does not have adverse vasopressor effect. Two types are in final stages of Phase III clinical trial and one of these approved for human use in South Africa.
conjugated Hb	PEG molecules are linked to each Hb molecules. PEG plus water of hydration results in conjugated Hb of larger dimension.	If each of the PEG-Hb with its added water of hydration approaches the required nanodimension, then there is no vasopressor effect as long as there is no free Hb molecules. In ongoing Phase II clinical trial
Xlinked molecular Hb	Each Hb molecule is intramolecularly crosslinked to prevent the Hb from breaking down into half molecules (dimers)	These molecular dimension Hb cross the intercellular junction of the endothelial cell lining of blood vessels and removes nitric oxide needed for normal vasoactivity.
Recombinant molecular Hb	Recombinant human Hb with fusion of the two α subunits of each Hb molecule to prevent its breakdown into half molecule (dimers).	Vasopressor effect observed in clinical trial for the same reason as above. A new recombinant human Hb has been prepared that does not bind nitric oxide thus obviating the problem with vasopressor effect. It is still remove faster from the circulation but it is a potential source of Hb for polyHb and conjugated Hb and other future generation Hb based blood substitutes

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[19]. 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. Opponents to the hypotheses argue that one cannot compare the different types of modified Hb since there are major differences in the chemistry involved and in the oxygen affinity. We therefore 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[24]. The result shows that the one with the lowest % of unpolymerized Hb molecules does not cause vasoconstriction nor changes in electrocardiogram. With increasing % of unpolymerized Hb molecules, there was increasing degree of vasoconstriction and elevation of the ST segment of the electrocardiogram. 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[23]. Further support of the importance of nanobiotechnology based polyHb has come from one group preparing large "zero-link" bovine polyHb[25]. A second generation molecular dimension

recombinant Hb that did not bind nitric oxide, also did not cause vasoconstriction[11]. Since recombinant Hb crosses the intercellular junction and is removed quickly, their circulation time can be increased by crossliking to form polyHb . Another approach in ongoing phase II clinical trial is to prepare PEG conjugated Hb that with its water of hydration would result in a sufficiently large modified Hb that would not cross the intercellular junction[10,17,26].

Research into 2nd generations rbc substitutes based on nanotechnology

First generation polyHb or conjugated Hb are suitable for several important clinical applications. There also have a number of advantages when compared to rbc (Table II).

TABLE II : comparing donor blood and first generation blood substitutes.

	Human donor blood	**Nanobiotechnology based polyHb and conjugated Hb**
Infective agents	Rare in some regions using costly screening tests, but more frequent in other regions. If new unknown infective agent appears it may take years to develop screening test for blood to be safe	Infective agents can be sterilized and removed.
Source	Limited availability	Unlimited : Since in addition to human Hb (Hb), bovine Hb and recombinant human Hb can also be used.
Blood group antigens	Yes and thus need typing and cross-matching.	No blood group antigens
Delay for use	Delay in use due to need for typing and cross matching	Can be used on the spot since no typing or cross matching needed. Being tested in ambulance in ongoing Phase III clinical trial.
Storage stability	Can be stored for about 42 days with standard refrigeration storage at 4^{o}	Studies show that polyHb can be stored for more than 1 year at room temperature
Circulation time in the body after infusion	About 60 days depending on the length of storage	Only circulate effectively for about 1 day and is therefore good for short term use as for surgery and in emergency. However, there are ways to increase its length of function. Hemodilution is one way. Also, infusion can be repeated a few times and it is also possible to combine with erythropoietin.
Function	Complete red blood cell (rbc) functions. Thus in addition to carrying oxygen, rbc also has antioxidant enzymes and enzymes for preventing metHb formation.	Function only as an oxygen carrier and is useful for a number of clinical applications. For some other applications, may require new generations of PolyHb (e.g. PolyHb crosslinked to antioxidant enzymes). Future generation nano artificial rbc contains Hb and all the enzyme systems of rbc. Cost will increase with each new generation.
Vasopressor effects	No vasopressor effects	To prevent vasopressor effects molecular dimension Hb has to be eliminated

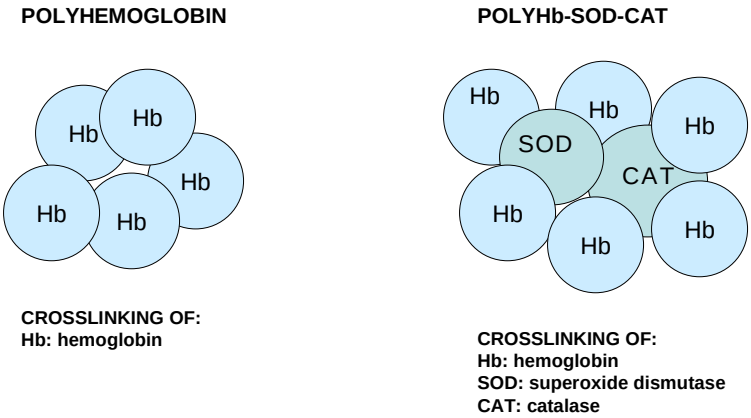
However, because they are only oxygen carriers and do not have the same enzyme activity as red blood cells that might be needed for some important clinical applications. As a result, new generations of blood substitutes are being studied[27]

PolyHb crosslinked with antioxidant enzymes.

As polyHbs can be kept at room temperature and used immediately on the spot, they could have considerable potential for treating severe haemorrhagic shock. However, Hb is a reactive molecule[28] and if there is substantial delay and if the haemorrhagic shock is severe, reperfusion using an oxygen carrier alone might result in ischemia reperfusion injuries owing to the production of oxygen radicals.

We have prepared a new generation of polyHb based on crosslinking polyHb with superoxide dismutase and catalase (PolyHb-SOD-CAT) (Fig. 2) [29-31]. This can transport oxygen and at the same time remove oxygen radicals so as to lessen the effects of ischemia-reperfusion injuries.

POLYHEMOGLOBIN-SUPEROXIDE DISMUTASE-CATALASE



The intestine is one of the organs that is most likely to be injured by ischemia-reperfusion in sustained severe hemorrhagic shock. We found that PolyHb-SOD-CAT, unlike PolyHb, did not cause a significant increase in oxygen radicals when it was used to reperfuse ischemic rat intestine[30]. Another example is in the obstruction of arteries owing to thrombosis or other causes that can result in stroke, myocardial infarction and other conditions. PolyHb and conjugated Hb are in solution and are therefore more likely to perfuse partially obstructed vessels compared with red blood cells. However, if arterial obstruction and lack of oxygen is prolonged, reperfusion with an oxygen carrier alone might also result in ischemia reperfusion injuries. Thus we found that in a rat stroke model, after >60 minutes of ischemia, reperfusion with polyHb resulted in significant

increase in the breakdown of the blood-brain barrier and an increase in brain oedema[31]. However, polyHb-SOD-CAT can supply oxygen without causing these adverse changes[31.] (Figures 1 and 2) Another way to prevent ischemia-reperfusion injury is the use of polynitroxylated cross-linked Hb[32]

PolyHb crosslinked with tyrosinase.

Malignant tumours are well vascularized but have abnormal microcirculation, resulting in under-perfusion by red blood cells and therefore lower tissue oxygen tension. Attempts have been made to decrease the blood supply even further by the use of agents that inhibit angiogenesis to prevent the growth of blood vessels to the tumour, but radiation therapy and chemotherapy work better with better tissue oxygen tension. As polyHb is in solution, it can perfuse the abnormal microcirculation of tumours more effectively than red blood cells to supply more oxygen for radiation therapy[33]. Furthermore, as polyHb stays in the circulation for only 24 hours, it has a short duration of action and so only functions during radiation therapy or chemotherapy. PolyHb decreases the rate of growth and increase the lifespan in a rat model of gliosarcoma brain tumour when used as an adjunct for chemotherapy[6.] We have recently crosslinked tyrosinase with Hb to form a soluble nanodimension polyHb-tyrosinase complex[34-35]. This has the dual function of supplying the oxygen needed for optimal chemotherapy or radiation therapy and also lowering the systemic levels of tyrosine. We have shown that , intravenous injections of polyhemgolboin-tyrosinase can delay the growth of the melanoma without causing adverse effects or changes in the growth of the treated animals[35]. Intravenous injections of polyhemgolobin-tyrosinase can be combined with oral administrations of microencapsulated tyrosinase to maintain a low systemic tyrosine level[36].

NANOBIOTECHNOLOGY TOWARDS A COMPLETE 3RD GENERATION RED BLOOD CELL SUBSTITUTE

In the first and second generation rbc substitute described above, Hb is in direct contact with blood and therefore needs to be highly purified to remove trace contaminants. Furthermore, Hb outside the red blood cell is more reactive and has a short circulation time: therefore is useful only for short-term applications. Attempts have been made to study third generation red blood cell substitutes with all the contents of rbc but replacing the rbc membrane with synthetic membrane. Such artificial cells would resemble red blood cells more closely than polyHb alone and all the enzymes found in red blood cells can be included. The surface properties of such an artificial cell can also be modified to increase their circulation time. In addition to Hb specially prepared cross-linked Hb or recombinant Hb can also be encapsulated inside these artificial cells.

Artificial red blood cells containing Hb and enzymes

Microencapsulation of the contents of red blood cells, including Hb and enzymes, inside artificial red blood cells with artificial membranes was first reported by this author in 1957 (www.artcell.mcgill.ca)[2,19] This system has an oxygen-dissociation curve comparable with that of red blood cells. Red-blood-cell enzymes such as carbonic anhydrase[2] and catalase[37] in these artificial red blood cells continue to function. For example, artificial cells containing Hb and catalase have been used to replace the antioxidant functions in mice with an inborn defect in red-

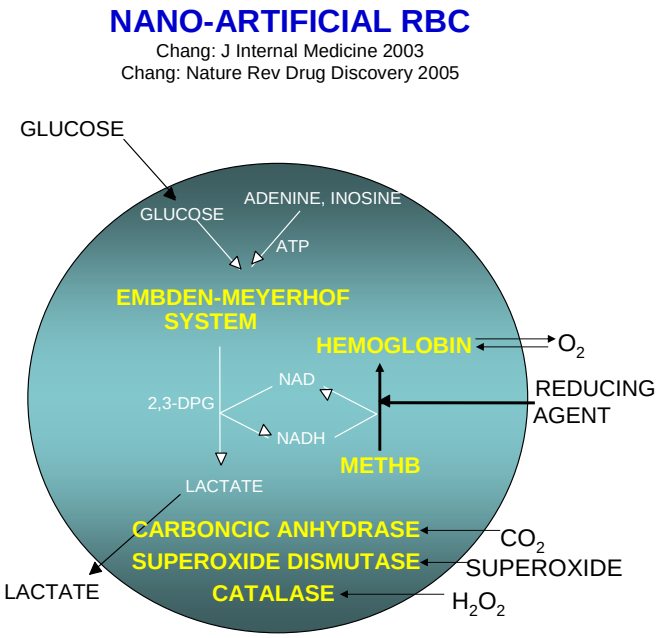
blood-cell catalase[37]. The major problem was a short circulation time resulting from rapid uptake by the reticuloendothelial system[18]. Use of membranes with different surface properties including negative surface charge and polysaccharides such as sialic-acid analogs[2,18] only resulted in a small but significant increase in circulation time. This has led to the next step in artificial red blood cell development.

Hb lipid vesicles

In 1980, small lipid-membrane artificial red blood cells approximately 0.2 μm in diameter were prepared using lipid-membrane liposomes[38] and found to remain in the circulation for a longer period than those described above. Extensive research on bilayer-lipid membrane artificial red blood cells has been carried out. Polyethylene(PEG)-lipid vesicles are especially effective in increasing the circulation time[39] PEG-lipid Hb lipid vesicles have been developed and scale up for preclinical animal studies[20,40] with promising results. Detailed safety and efficacy studies are being carried out towards eventual clinical trials[20,40]. The potential shortage of Hb has led this group to also study the incorporation of synthetic heme into lipid vesicles or to recombinant albumin to avoid the need for using Hb[41]. They have also linked synthetic heme to recombinant albumin to form an oxygen carrier[41]

Biodegradable-copolymer membrane nano artificial red blood cells containing Hb and red blood cell enzymes

We are studying a new approach combining nanobiotechnology with biodegradable polymer to decrease effects on the reticuloendothelial system and increase Hb loading. A biodegradable polymer, polylactide (PLA) is degraded in the body into lactic acid, then water and carbon dioxide and has been used in implants with no side effects. We also have experience in preparing microscopic dimension artificial cells using PLA, having initiated this in 1976[42] Thus, more recently, we have prepared these in the nanodimension of 80-180 nanometer with a membrane thickness is 5 - 15 nm (Fig 3)[34-45]. Hb loading is normally 10.97 gm/dl but can be up to 15 gm/dl (as in normal blood) in a 50% suspension with only 1.2 gm of PLA. Thus these are not the sample as nanoparticles used for drug delivery where most of the nanoparticle consist of the polymeric material.



Oxygen dissociation curve of the preparation containing 10.97 gm/dl bovine hemoglobin is not significantly different from free bovine hemoglobin. Hill coefficient is 2.4 to 2.9. These results show that the procedure of preparation does not have adverse effects on the hemoglobin molecules. Polylactide is degraded into lactic acid and then water and carbon dioxide . For a 500 ml suspension, the total lactic acid produced is 83 mEq. This is far less than the normal resting body lactic acid production (1000-1400 mEq/day). The maximal body capacity to breakdown lactic acid is 7080 mEq/day. Thus, 83 mEq is equal to 1% of this. Our preliminary histological studies show that the ultrathin PLA membrane is rapidly biodegraded and there is no accumulation in the reticuloendothelial system.

This type of rbc substitute contains Hb and all the rbc enzyme system Superoxide dismutase catalase and metHb reductase (Fig 3)[34-45].Similar to red blood cells, Hb is being continuously converted into the non-oxygen carrying form of metHb, but the metHb reductase system can enzymatically convert metHb to Hb since nanocapsules are permeable to glucose and other small hydrophilic molecules[44,45]. Reducing agents from plasma can also diffuse into the nanocapsules to convert metHb to Hb as has been demonstrated by *in vitro* studies[45]. To increase the circulation time, we synthesized several new polyethylene glycol(PEG)-polylactide(PLA) copolymers for the membrane of the nano artificial red blood cells [45]. Infusion of 1/3 the blood volume into rats did not result in vasopressor effects. One of these, when used for the membrane of nanocapsules can markedly increase the circulation time of the nanodimension artificial red blood cells. When we use the rat results in this study for polyHb and its clinical equivalent in human of about 24 hours, the best PEG-PLA Hb nanocapsules is equivalent to about minimal of 41.5 hours in human. This is likely to be even higher compared with PolyHb because the reticulo-

endothelial systems in rats are much more efficient than human in removing particulates like nanocapsules as compared with PolyHb solution. These PEG-PLA nanodimension artificial red blood cells could be useful carriers for other modified Hb including recombinant Hb, PEG conjugated Hb to prolong further their circulation time, inclusion of enzyme systems and avoid direct external exposure.

Discussions

Regional differences and the potentials of unknown infective agents must be included in any discussions of the future prospect of blood substitutes and also in the degree of regulatory requirements. Blood substitute is urgently needed in regions of the world where there are severe shortage of donor blood because of cultural or religious believes that made it less willing for people to donate blood. It is also urgently needed in regions with higher incidences of infective agents such as HIV and thus higher potential for contaminated donor blood. It is less urgent in regions with less incidences of HIV and where costly screening tests are being used to screen out infective agents in donated blood. However, it is important to remember the past unexpected outbreak of HIV and hepatitis C and the resulting contaminated donated blood that persisted for years until proper screening tests were developed. If this should happen again with some yet unknown agents, (e.g. avian flu and others) then it would again be disastrous if no blood substitutes, even first generation blood substitutes, is immediately available. Past experience has shown that it takes many years to develop ideas on blood substitutes into products and that lack of basic information has resulted in much failure and delays. It is important to carry out basic research to gain important basic information needed for the simultaneous development of blood substitutes. In the meantime, two types of first generation nanodimension polyHb are in the final stages of clinical trials in human and one of these has been approved for routine clinical uses in patients in South Africa. New nanodimension conjugated Hb is also being tested in clinical trial. Shortage of human Hb is being resolved by studies on recombinant human Hb, placenta Hb , bovine Hb and synthetic heme. Meanwhile, new generations of modified Hb are being developed that can modulate the effects of nitric oxide for those clinical applications that might have potential problems related to oxygen radicals. PolyHb can be crosslinked to an enzyme to suppress the growth of tumour. A further development is the use of PEG-lipids or PEG-biodegradable polymer membranes to prepare nanodimension artificial red blood cells containing Hb and complex enzyme systems.

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