CHAPTER 1

- **UNLIMITED POTENTIAL OF NANOBIOTECHNOLOGY BASED BLOOD SUBSTITUTES, ARTIFICIAL CELLS AND NANOBIOTHERAPEUTICS**
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1. INTRODUCTION

1.1.Why do we need blood substitutes?

Under normal circumstances, donor blood (rbc) is the best replacement for blood (Yang et al 2017). HOWEVER, as quoted from an editorial (Chang 2017):

- Natural epidemics (e.g. HIV, Ebola etc) or man-made epidemics (terrorism, war, etc) can result in contaminated donor blood or disqualified disease contact donors. Unlike rbc, blood substitutes can be sterilized.
- Heart attack and stroke are usually caused by obstruction of arterial blood vessels. Unlike rbc particles, blood substitute is a solution and in animal studies it can more easily perfuse through obstructed vessels to reach the heart and brain.
- Severe blood loss from accidents, disasters or war may require urgent blood transfusion that cannot wait for transportation to the hospital for blood group testing. Unlike rbc, blood substitutes do not have blood groups and can be given on the spot (Fig. 8).
- Red blood cells have to be stored in refrigeration for up to 42 days thus difficult to transport and store in disaster and frontline. Blood substitutes can be stored at room temperature for more than 1 year, compared to rbc of 1 day at room temperature.
- 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. Animal study shows that one type of blood substitutes with enhanced rbc enzymes can prolong the time.

1.2. Why do we need Nanobiotechnology for blood substitutes?

Hemoglobin, a tetrameric protein, is responsible for the transport of oxygen in red blood cells (Perutz, 1980). Attempts to use hemolysate (Amberson, 1937) and stroma-free hemoglobin as oxygen carrier (Rabiner, 1967) resulted in nephrotoxicity and cardiovascular adverse effects (Savitsky, 1978). The small tetrameric hemoglobin molecule causes much of the problem and is made worse since once infused the tetramer breaks down into even more toxic dimers.

2.COMPLETE ARTIFICAL RED BLOOD

The first artificial red blood cells that contain hemoglobin and red blood cell enzymes (Fig. 1) have oxygen dissociation curve similar to red blood cells (Chang, 1957, 1964). Hemoglobin stays inside as tetramers and red blood cell enzymes like carbonic anhydrase and catalase retain their activities (Chang, 1964, 1972). These artificial red blood cells do not have blood group antigens on the membrane (Figure 1) and therefore do no aggregation in the presence of blood group antibodies (Chang, 1972). However, the single major problem was the rapid removal of these artificial cells from the circulation. Nanobiotechnology based soluble complex was therefore investigated to increase the circulation time.

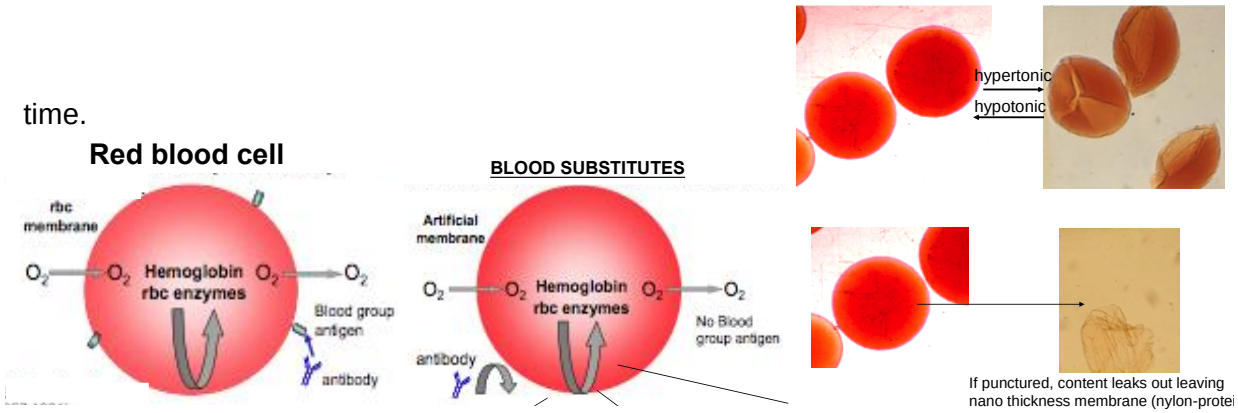

Figure 1: *Left red blood cells* ***Middle*** *Artificial red blood cells schematic.* ***right:*** *Artificial red blood cells of microscopic dimensions that can reversibly "crenate" in hypertonic solution. Updated from Chang (Chang 1965, 2007) with copyright permission*

After the first reports of artificial red blood cells in 1957, 1964 (Chang 1957, 1964) people felt that blood substitute is a simple matter that could be quickly developed when needed. Thus, blood substitute research was put aside and only the other areas of artificial cells were extensively developed around the world for other wide-spread uses (Chang 1972, 2005, 2019). When AIDS arrived in 1989 there was no blood substitutes and many patients were infected with H.I.V. contaminated donor blood. It is only then that intense R&D on blood substitutes was belatedly carried out around the world. It was found out too late that blood substitute requires the same long-term research as in any other medical research for cancer and other diseases.

Red blood cells have 3 major functions: (1) transport oxygen from the lung to the tissue, (2) remove damaging oxygen radicals and (3) carry carbon dioxide CO2. from the tissue to the lung to be removed. The urgency of H.I.V. in donor blood necessitates the development of the simplest system in the shortest time in the form of one of the 3 red blood cell functions, oxygen carrier.

3. NANOBIOTECHNOLOGY BASED OXYGEN CARRIERS

3.1.Basic principle

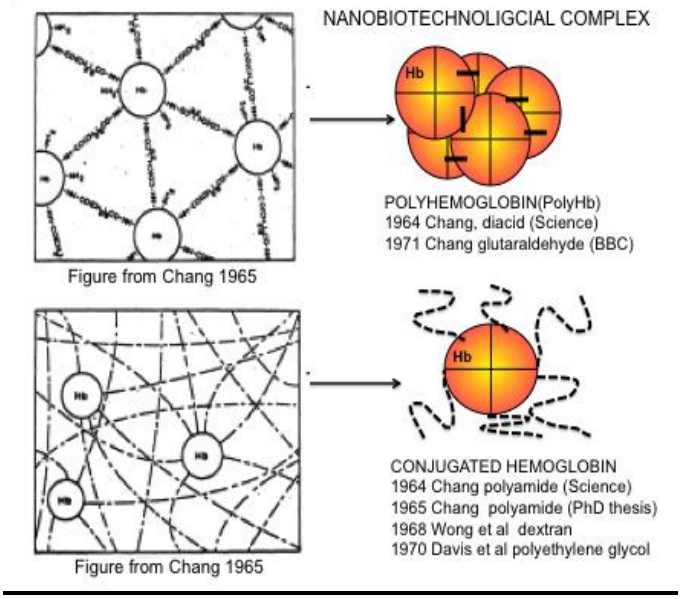

Figure 2 *Basic principle of polyhemoglobin and conjugated hemoglobin (Chang 1964, 1965)*

The original micro dimension artificial red blood cells are too large to survive in the circulation Thus modified hemoglobin was developed. These are based on the Chang's earlier basic principle (Chang 1964,1965,1971) of polyhemoglobin and conjugated hemoglobin (Figure 2).

The four types of most commonly studied first generation hemoglobin based oxygen carriers are shown in Figure 3 These are polyhemoglobin, conjugated hemoglobin, intramolecularly crosslinked tetrameric hemoglobin and recombinant hemoglobin. Unlike red blood cells, there is no blood group, and thus can be given on the spot, without waiting for typing and cross-matching in the hospital. They can be sterilized and are thus free from infective agents such as HIV, hepatitis C, bacteria, parasites and so on. Whereas donor blood has to be stored at 4°C and is only good for 42 days, modified hemoglobin can be stored at room temperature for more than one year.

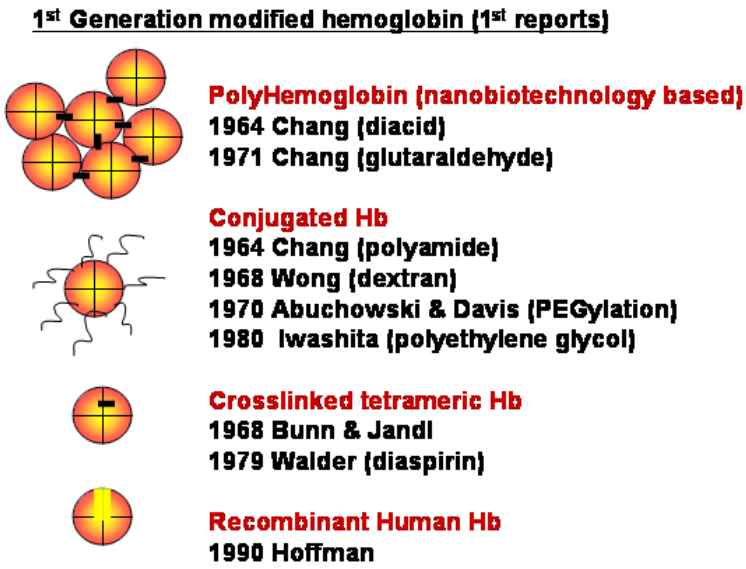

Figure 3. *The four types of most commonly studied first generation hemoglobin based oxygen carriers*

3.2.Polyhemoglobin for use as oxygen carrier

The 1971 basic principle of glutaraldehyde crosslinked polyhemoglobin (PolyHb) (Chang 1971) (Fig.2,3) has been independently developed most extensively by centers around the world (Dudziak & Bonhard, 1980, DeVent0 & Zegna, 1982, Keipert, Minkowitz & Chang, 1982, Keipert & Chang, 1987 ,Sehgal et al, 1983, Feola et al 1983, Moss et al 1988, Gould et al, 1995,. Pearce & Gawryl, 2006, Jahr et al 2008, Greenburg et al 2008

3.3.Glutaraldehyde crosslinked human PolyHb:

Gould and Moss started the Northfield Laboratory to develop glutaraldehyde crosslinked human PolyHb (Sehgal et al, 1983, Moss et al 1988, Gould et al, 1995). Their clinical trial on 171 patients shows 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 (Gould et al 2002).. In 2008 they reported their multicenter randomized clinical trial on in pre-hospital ambulance patients. Since no typing and cross-matching is needed and it can be used right on the spot. Their result in about 700 hemorrhagic shock patients shows that PolyHb can maintain the patients for 12 hours after reaching the hospital. In the saline control group, most of the patients need blood transfusion shortly after reaching the hospital (Moore et al, 2008).

3.4.Glutaraldehyde crosslinked bovine PolyHb:

Bing L. Wong and Carl Rausch were the cofounders of Biopure to start work on Glutaraldehyde crosslinked bovine PolyHb. Recent overviews of the development and extensive clinical trials are available (Pearce & Gawryl, 2006, Jahr et al 2008, Greenburg et al 2008, Greenburg 2013). For example, they have carried out 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. South Africa and Russia have approved this for routine clinical uses in patients. Mer et al (2016) discusses Hemoglobin glutamer-250 (bovine) in South Africa consensus usage guidelines from clinician experts who have treated patients.

3.5.Other sources of hemoglobin for PolyHb

In addition to hemoglobin from outdated human donor blood, bovine Hb, as mentioned above is another source (Feola et al 1983). Other sources of hemoglobin have also been used for PolyHb. These included, for example, preclinical studies on porcine Hb (Zhu et al 2007, Zhu and Chen 2013) and Hb from human placental blood (Li et al 2006). These two groups have carried out extensive laboratory and preclinical studies. Other possible sources of hemoglobin include recombinant hemoglobin (Hoffman et al 1990), marina Hb (Rousselot et al 2006) and Bulow others.

3.6.Conjugated hemoglobin

In the presence of diamine, sebacyl chloride crosslinks hemoglobin with polyamide to form conjugated hemoglobin [Chang 1964,1965] (Fig.2). An extension of this is the crosslinking of single hemoglobin molecule to soluble polymers like dextran [Wong et al 1988 Tam, Blumenstein & Wong, 1976)] or PEG (Abuchowski et al, 1977, Iwashita, 1992, Yabuki et al 1990, Shorr, Viau & Abuchowski, 1996, Li, Zhang & Liu, 2005, Winslow 2006 , Liu and Xia 2008, Seetharama et al 2013) (Fig. 3). PEG-Hb shares many of the advantages of PolyHb as described above. More details are available in other later chapter. Clinical trials have been carried on PEG-Hb [Winslow, 2006, Li, Zhang & Liu, 2005, Liu and Xia 2008]. Keipert meeting in Montrea Later phases of clinical trials on PEG-Hb have not yet been published.

3.7.Intramolecularly crosslinked hemoglobin and recombinant hemoglobin

In addition to glutaraldehyde crosslinked PolyHb and conjugated Hb there are other ways of modifying hemoglobin (Fig.3). These include intramolecularly crosslinked tetrameric hemoglobin (Walder et al, 1979, Przybelski et al 1966, Burhop & Estep, 2001), recombinant human hemoglobin (Looker et al, 1992, Shoemaker et al, 1994). Some have resulted in adverse effects like vasoconstriction in clinical trials. This has led to the proposal that the intercellular junctions of the endothelial lining of vascular wall allow tetrameric 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. However, this can be avoided if nitric oxide removal is prevented by a specially designed recombinant Hb (Doherty et al, 1998) or a modified form of stabilized intramolecularly crosslinked Bovine Hb (Wong et al 2011) or by the administration of nitric oxide (Yu et al 2010, Zapol 2012)

3.8.Other effects

Those polyhemoglobin or conjugated Hb that contain high levels of uncrosslinked hemoglobin or low molecular weight PolyHb could have adverse effects (Kim and Greenburg 1997, Chang,1997, 2007, Bucci, 2011, 2013)(Fig 4). There are also other factors including pathological characteristics of patients, like endothelial dysfunction (Yu et al 2010). Furthermore, the design of preclinical and clinical study is complicated (Greenburg & Kim, 1992. Zuck, 1994, Fratantoni, 1994, Klein, 2000, Chang, 1997, 2007, Winslow, 2006, Greenburg & Kim,1992, Greenburg et al 2008, Greenburg 2013). As mentioned earlier, vasoconstriction can be avoided if nitric oxide removal is prevented by a specially designed recombinant Hb (Doherty et al, 1998) or a modified form of stabilized intramolecularly crosslinked Bovine Hb (Wong et al 2011) or by the administration of nitric oxide (Yu et al 2010, Zapol 2012). Thus, one cannot attempt to combine the clinical trial results of different types of hemoglobin based blood substitutes and different clinical conditions into a single meta-analysis as has been done (Natanson et al 2008)

In medicine, nothing can be considered to be a “cure all”. First generation hemoglobin oxygen carriers are more suitable for some clinical conditions especially in patients with no endothelial dysfunction or no sustained ischemia or elevated tissue pCO2. However, new generations need to be developed to complement and supplement the first generation. On the other hand, there is no reason to use a more complicated and more expensive new generation system if the clinical conditions can be treated safely and effective using the first-generation ones.

4. NANOBIOTECHNOLOGY BASED OXYGEN CARRIERS WITH ANTIOXIDANT FUNCTI0NS

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Arterial obstruction can result in stroke and heart attack. Red blood cells, being 7 to 8 microns in diameter, have difficulty flowing through partially obstructed vessels to supply the needed oxygen. PolyHb, being a solution, can perfuse through to supply the needed oxygen. However, reperfusion with an oxygen carrier can release damaging oxygen radicals (Fig. 4).

D’Agnillo and Chang has prepared a soluble complex of Polyhemoglobin containing antioxidant enzymes to remove oxygen radicals (PolyHb-SOD-CAT) (D’Agnillo and Chang 1998). It has the dual function of an oxygen carrier that can also remove oxygen radicals (Fig. 4). In this form the SOD and CAT can be enhanced to be much higher than those in red blood cells.

After 90 min of combined hemorrhagic shock and brain ischemia in rats, reinfusion of PolyHb-SOD-CAT did not cause brain edema (Fig.4) (Powanda and Chang, 2002)). On the other hand, PolyHb or a solution contain free Hb, SOD and CAT causes significant increases in brain edema.

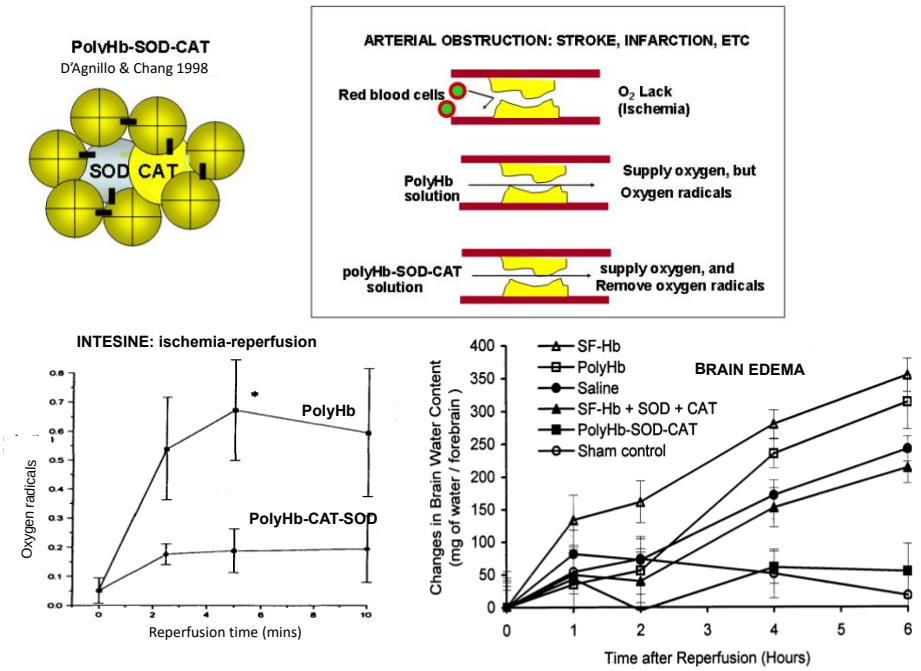

Figure 4. ***Upper left*** *PolyHb-SOD-CAT* ***Upper right:*** *Arterial obstruction can result in stroke and heart attack. Red blood cells cannot flow through. PolyHb, a solution, can perfuse through. (****Upper left****) PolyHb-SOD-CAT, a solution can perfuse through to supply oxygen and remove oxygen radicals* ***Lower right:*** *Unlike PolyHb, reinfusion of PolyHb-SOD-CAT does not cause brain edema in rat brain ischemia.* ***Lower left:*** *Unlike PolyHb, PolyHb-SOD-CAT reperfusion in ischemic small intestine does releases damaging oxygen radicals. (from Chang 2007 with copyright permission)*

Thus in a rat hemorrhagic shock-cerebral ischemia ratmodel, 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 water (brain edema) [Powanda & Chang, 2002]. On the other hand, PolyHb-SOD-CAT did not result in these adverse changes [Powanda & Chang, 2002]. (Fig.6). Ischemia-reperfusion injury in severe sustained hemorrhagic shock can result in damage to the intestine with leakage of E-coli or endotoxin to the systemic circulation resulting in irreversible shock. Thus, we studied the perfusion of isolated small intestine. in a rat model of intestinal ischemia reperfusion (Razack, D'Agnillo& Chang, 1997). and found that Ischemic small intestine releases damaging oxygen radicals when reperfused with PolyHb. However, PolyHb-SOD-CAT reperfusion did not increase oxygen radical release (Fig 4). This is important during intestinal surgery or organ storage for transplantation (Razack, D'Agnillo& Chang, 1997).

Others have used this for pancreatic beta cells in rats (Nadithe & Bae, 2011); for myocardial infarction attenuation in rats (Wang et al 2012) and rat kidney transplantation from Korea (Chang et al. 2004). Hsia extended the PolyHb-SOD-CAT approach to prepare a hemoglobin with synthetic antioxidant based on the convalently binding of nitroxides (Buehler et al 2004, Ma and Hsia, 2013). In another approach, using his background on the subject (Alayash, 2004), a Hb-haptoglobin complex can also be used to protect against oxidative stress (Jia and Alayash, 2013). Another one is Zal's Arenicola marina Hb (Rousselot et al 2006) with antioxidant activity. Simoni et al (1997) added a pharmacological solution with antioxidant function to their modified Hb.

5 NANOBIOTECHNOLOGY BASED OXYGEN CARRIERS WITH ENHANCED CO2 TRANSPORT AND ENHANCED ANTIOXIDANT FUNCTI0NS

5.1. Polyhemoglobin-superoxide dismutase-carbonic anhydrae

Sims et al (2001) used a novel microelectrode to measure tissue pCO2 in animal model of severe hemorrhagic shock. And reported that mortality is related to the elevation of tissue pCO2. Carbonic anhydrase (CA) in red blood cell is the major means for the transport of tissue CO2 to the lung. We therefore use the nanobiotechnological method to assemble CA with hemoglobin and antioxidant enzymes to form PolyHb-SOD-CAT-CA (Bian et al 2011, Bian & Chang 2015). (Fig. 5). It not only has all 3 rbc functions, but it can have enhancement of all 3 rbc functions by increasing the concentrations of rbc enzymes in the complex (Bian & Chang 2015). These rbc enzymes can be extracted from bovine rbc inexpensively (Guo, Glynn & Chang 2015). This complex has no blood groups.

5.2. Result in a 90 minutes sustained hemorrhagic shock rat model

Our result in a 90 minutes hemorrhagic shock animal model with 2/3 blood volume loss (Fig 10) shows that it is superior to whole blood in the lowering of elevated intracellular pCO_2 , recovery of ST elevation, tropronin levels, lowering of elevated lactate, histology of the heart and intestine. It is more efficient than red blood cell in a sustained 60 minutes hemorrhagic shock rat model (Bian et al 2013). It is even more effective in a 90 minute sustained hemorrhagic sock model (Bian & Chang 2015) (Fig. 5).

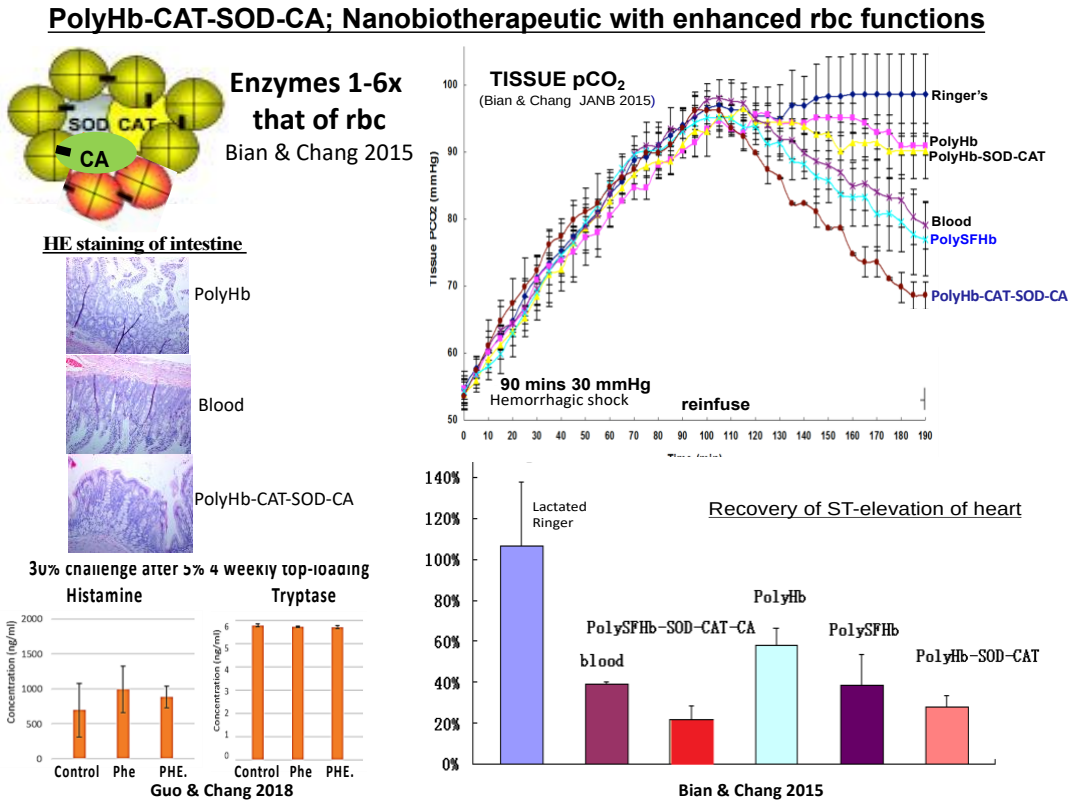

Figure 10: Upper left: *Polyhemoglobin-catalase-superoxide dismutase-carbonic anhydrase can have up to 6 times red blood cell enzyme concentration. In a rat hemorrhagic shock model with 2/3 blood volume loss and 90 mins sustained shock the result is as follow:* ***Upper right:*** *significant faster lowering of the elevated tissue pCO_2 and* ***Lower Right:*** *faster recovery of the ischemic heart* ***Middle right:*** *intestine having better histological finding.* ***Lower left:*** *Test for anaphylactic reaction: no significant increase in tryptase nor histamine. Above figures from Chang's group (29,32,36)*

5.3. Safety and Immunology Long term study of bovine PolyHb-SOD-CAT-CA was carried out in rats. This consisted of 4 weekly 5% blood volume infusion followed by 30% volume exchange transfusion (Guo & Chang 2018))**.** The result showed safety and lack of immunological problems. This includes the measurement of histamine and tryptase that show no anaphylactic reaction (Fig. 5). Hemoglobin has very low antigenicity. Bovine PolyHb itself shows no immunological problems in patients (Kim & Greenburg 2014). For PolyHb-SOD-CAT-CA the small fraction of enzymes are nanoencapsulated inside the large excess of hemoglobin molecules (Guo & Chang 2018) (Fig.5)

5.4. Stability

What is the stability of the enzymes in this complex? Unlike PolyHb, the enzyme component may not be sufficiently stable with storage especially in room temperature and hot climate. We found that the complex can be lyophilized, freeze dried. Unlike about 1 day for rbc at room temperature, this lyophilized preparation can be stored in room temperature for 320 days (Bian, Guo & Chang 2016). The freeze-dried powder preparation of PolyHb-SOD-CAT-CA is stable for more than 360 days at 4C as compared to 40 days for donor blood. . The lyophilized preparation can be heat pasteurized at 68F for 2 h (Bian, Guo & Chang 2016). This can be important if there is a need to inactivate H.I.V. virus, Ebola virus, COVID-19 and other infective organisms. The freeze-dried powder is much easier for storage since it takes up little space also being very light and compact, it is easy for transportation. This is especially important for emergency, space travel, disasters or war.

6. NANODIMENSION COMPLETE ARTIFICIAL RED BLOOD CELLS.

6.1. Early artificial red blood cells.

The first artificial red blood cells (Fig. 1) have all the in vitro function of red blood cells as shown by oxygen dissociation curve (Chang, 1957), carbonic anhydrase activity (Chang, 1964) and catalase activities (Chang & Poznansky, 1968). These artificial red blood cells do not have blood group antigens on the membrane and therefore do no aggregation in the presence of blood group antibodies (Chang, 1972). However, the single major problem is the rapid removal of these artificial cells from the circulation. Much of the studies since that time are to improve survival in the circulation by decreasing uptake by the reticuloendothelial system. Since removal of sialic acid from biological red blood cells resulted in their rapid removal from the circulation (Chang 1965,1972), we started to modify the surface properties on artificial red blood cells. This included synthetic polymers, negatively charge polymers, crosslinked protein, lipid-protein, lipid-polymer, addition of surface polysaccharides and others (Chang, 1965, 1972). Artificial red blood cells have since been extensively explored by many researchers around the world. These include Beissinger, Bian, Chang, Farmer, Gao, Hunt, Kobayashi, Lee, Mobed, Nishiyia, Rabinovic ,Rudolph ,Sakai, Schmidt, Sinohara, Szebeni, Takeoka, Tsuchida, Takahashi, Usuba and many others.

6.2. Bilayer lipid membrane nano artificial rbc

Bangham (Bangham et al 1965) reported the preparation of liposomes each consisting of microspheres of onion like concentric lipid bilayers for basic membrane research. The multi-lamellar liposome limits the amount of water-soluble drugs that can be enclosed. Thus, the basic principle and method of preparing artificial cells using ether (Chang, 1957, 1964) was extended into what they call an “ether evaporation method” to form single bilayer lipid membrane liposomes for drug delivery (Deamer and Bangham,1976). This was extended for the preparation of submicron lipid membrane artificial rbc (Djordjevich & Miller, 1980, Famer et al 1988, Phillips, Rudolph and Klipper, 1992, Rudolph, 1994, Kobayashi et al, 2005, Tsuchida et al 2006, Sakai, 2013). The circulation half time has been increased to 36 hours in rats by the addition of polyethylene glycol to the lipid membrane (Philips et al, 1999). These advances make it now possible to scale up for detailed preclinical studies towards clinical trial (Tsuchida, 1998, Kobayashi et al , 2005 Sakai, 2013). It is possible to replace 90% of the red blood cells in rats with these artificial red blood cells and it is also effective in hemorrhagic shock (Tsuchida, 1998, Kobayashi et al , 2005 Sakai, 2013). More updates and details will be available in later chapters in this book (Sakai 2020).

6.3. Nano-dimension biodegradable polymeric artificial cells

Using a modification of this author’s method of micron dimension biodegradable polymeric membrane artificial cells (Chang, 1976) we have prepared nano dimension PLA artificial red blood cells (Chang, 1997,2007, Chang et al 2003, Yu & Chang, 1996). This decreases the amount of lipid needed for the nano-artificial cells (Figure 6). Polymer membrane is stronger than bilayer lipid and a thinner polymer membrane can be used. Figure 6 compares the amount of membrane material in Hb

lipid vesicles compared to PLA nano-artificial red blood cells. Furthermore, unlike lipid membranes it is permeable to water soluble small and middle range molecules.

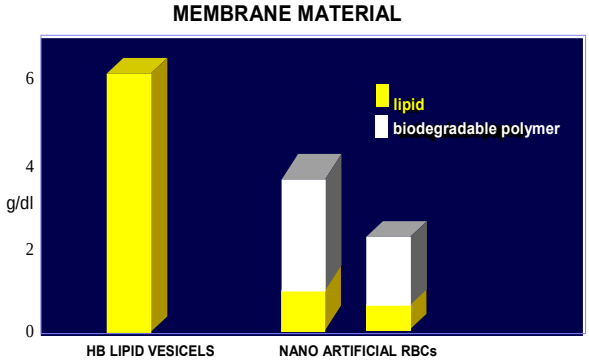

Fig. 6. Amount of membrane material in Hb lipid vesicles compared to PLA nano rbc's,. (With copyright permission from Chang 2007 Monograph on Artificial Cells)

Polylactide membrane in PLA nano rbc's is biodegradable into lactic acid and finally water and carbon dioxide and thus is not retained in the reticuloendothelial system.

These nano artificial RBC of 80 to 150 nanometers contain all the red blood cell enzymes and can convert methemoglobin to hemoglobin (Chang et al, 2003) (Fig. 7). The membrane is not permeable to large molecules, but freely permeable to small molecules like glucose and reducing agents from plasma. In vitro study shows that when incubated at 37◦C metHb increases quickly (Fig.13). Addition of a reducing agent, ascorbic acid prevents the increase in MetHb. Addition of glucose and NADH allows the Embden Meyerhof enzyme system in the nano artificial rbc to decrease MetHb further.(Fig.7)

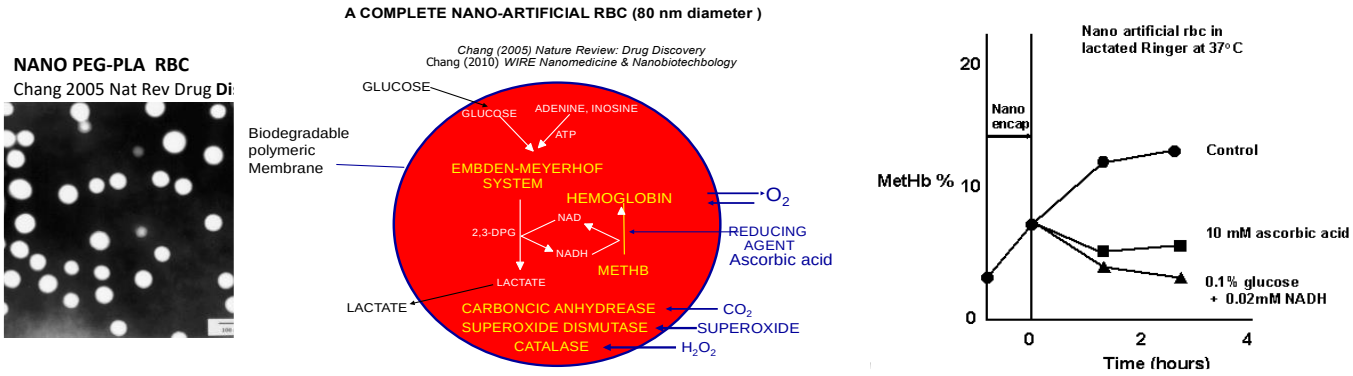

Fig:7 ***Left:*** *E/M photo of nano-dimension PEG/PLA artificial RBC* ***Middle*** *Biodegradable polymeric membrane nano artificial rbc contains hemoglobin and all the enzymes of rbc. The membrane is not permeable to larger molecules, but freely permeable to glucose and reducing agents from plasma.* ***Right****: When incubated at 37◦C metHb increases quickly. Addition of a reducing agent, ascorbic acid prevents the increase in MetHb. Addition of glucose and NADH allows the Embden Meyerhof enzyme system in the nano artificial rbc to decrease MetHb further. (With copyright permission from Chang 2007)*

Our studies show that using a polyethylene-glycol-polylactide copolymer membrane we are able to increase the circulation time to double that of polyhemoglobin (Chang et al, 2003). The results of other groups support these findings (Zhang et al 2008, Sheng et al 2009). We also reported that infusion of 1/3 blood volume into rats did not have any adverse effects on the kidney (Liu & Chan. 2008a) or the liver (Liu & Chang 2008b) on a long term basis. Our more recent study uses PEG-PLA membrane nano artificial cells containing polyhemoglobin-catalase-superoxide dismutase-carbonic anhydrase in a hemorrhagic shock rat model with 2/3 of the blood removed. After one hour of hemorrhagic shock at 30mmHg, infusion of this preparation effectively resuscitated the animal and lowered the elevated tissue PCO2 (Wei, Bian and Chang 2013). More details will be presented

in later chapters

6.4. Variations in the membrane of nano artificial rbc

PEG-lipid vesicles are more like the lipid–polymer membrane artificial cells (Chang, 1972) and are no longer pure lipid vesicles. Discher's group (Photos et al., 2003) used self-assembling of block copolymers. Poly(ethylene glycol) (PEG) was the hydrophilic block and polyethylene or polybutadiene (PB) was the hydrophobic block. This significantly increased strength when compared to PEG–lipid membrane artificial cells. This so-call polymersomes are PEG-PB nano artificial rbc similar to PEG-PLA nano artificial rbc. Thus, polymeric membrane artificial cells have branched off into multilamellar liposome that then has evolved into lipid membrane artificial cells, then polymer-lipid membrane artificial cells, and finally back to the polymeric membrane artificial cells that are now called by different names including polymersomes, nanocapsules, nanoparticles, vesicles and others.

6.5. Nonfunctional or functional membrane

Nano dimension artificial red blood cells have a much higher total surface area than red blood cells. This also means that there is much more total membrane material. Thus, both PEG-lipid and PEG-polylactide nano red blood cells contain substantial amount of nonfunctional lipid or polymeric membrane. On the other hand, for soluble nanobiotherapeutic artificial rbc, PolyHb-SOD-CAT-CA, the "membrane" is functional in the form of oxygen carrying hemoglobin (Fig.8).

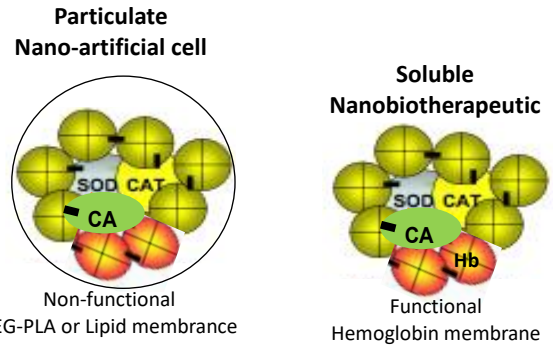

FIG 8. ***Left:*** *nano rbc contains substantial amount of nonfunctional lipid or polymer membrane.* ***Right****: Soluble nano rbc in the form of Hb complexed with enzymes have functional oxygen carrying hemoglobin as the "membrane". Updated from Chang (2017) with copyright permission*

7. NANOBIOTECHNOLOGY BASED OXYGEN CARRIERS WITH PLATELET FUNCTI0NS

PolyHb can replace the hemoglobin level in very severe hemorrhage, but in very severe blood loss, platelets also needs to be replaced since without platelets blood cannot clot. We use nanobiotechnology to assmble hemoglobin with fibrinogen to form PolyHb-fibrinogent (Wong and Chang, 2007).

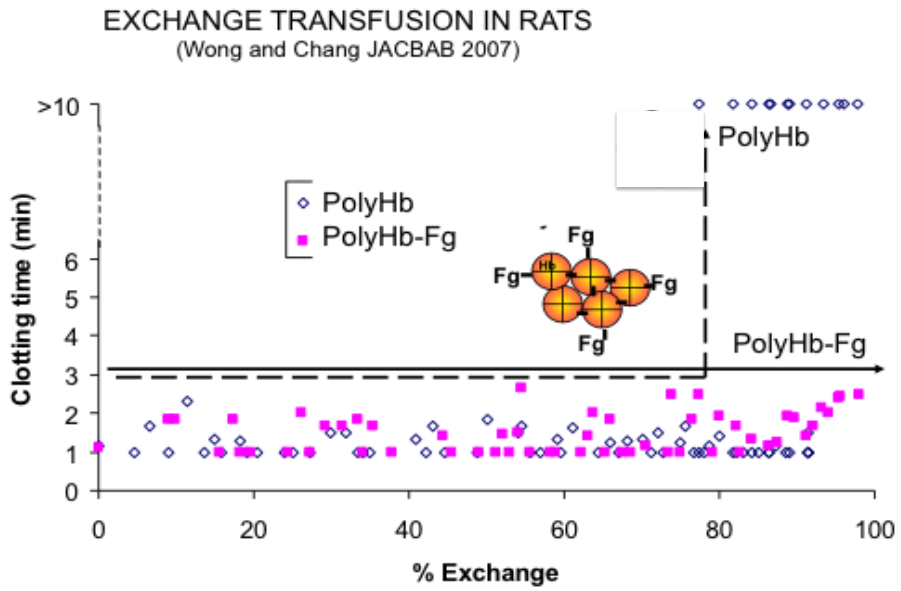

Figure 8. Exchange transfusion in rats. There is clotting problem when more than 80% of blood has been exchanged with PolyHb. There is no problem with clotting when 98% of the blood is replaced with PolyHb-fibrinogen with platelet-like activity. (From Wong and Chang, 2007)

We studied this in a rat model and found that replacing more than 80% of the total blood volume with PolyHb leads to defects in blood clotting (Wong and Chang 2007) (Fig. 9). . Using this, we can replace up to 98% of the total blood volume with PolyHb-fibrinogen without causing clotting problems (Wong and Chang, 2007) (Fig. 8). More details will be discussed in a later chapter.

8. NANOBIOTECHNOLOGY BASED OXYGEN CARRIERS WITH CANCER SUPPRESSION FUNCTI0N

Abnormal microcirculation in tumour leads to decrease in perfusion by oxygen carrying red blood cells. PolyHb can more easily perfuse the abnormal microcirculation of tumours to supply oxygen needed for chemotherapy or radiation therapy (Robinson et al 1995, Teicher, 1995,) (Fig. 9). Thus, PEG conjugated hemoglobin has been used this way (Han et al 2012, Shorr, Biau & Abuchowski, 1996). PolyHb also decreases the growth of tumour and increases the lifespan in a rat model of gliosarcoma brain tumour [Pearce & Gawryl, 1998].

We have crosslinked tyrosinase with hemoglobin to form a soluble PolyHb-tyrosinase complex [Yu and Chang, 2004] (Fig. 9). This has the dual function of supplying the needed oxygen and at the same time lowering the systemic levels of tyrosine needed for the growth of melanoma. Intravenous injections delayed the growth of the melanoma without causing adverse effects in the treated animals [Yu & Chang, 2004] (Fig.9). Our more recent study includes the use of PLA and PEG-PLA membrane nano artificial cells containing polyHb-tyrosinase (Furstier and Chang, 2012, Wang and Chang 2012, 2016)

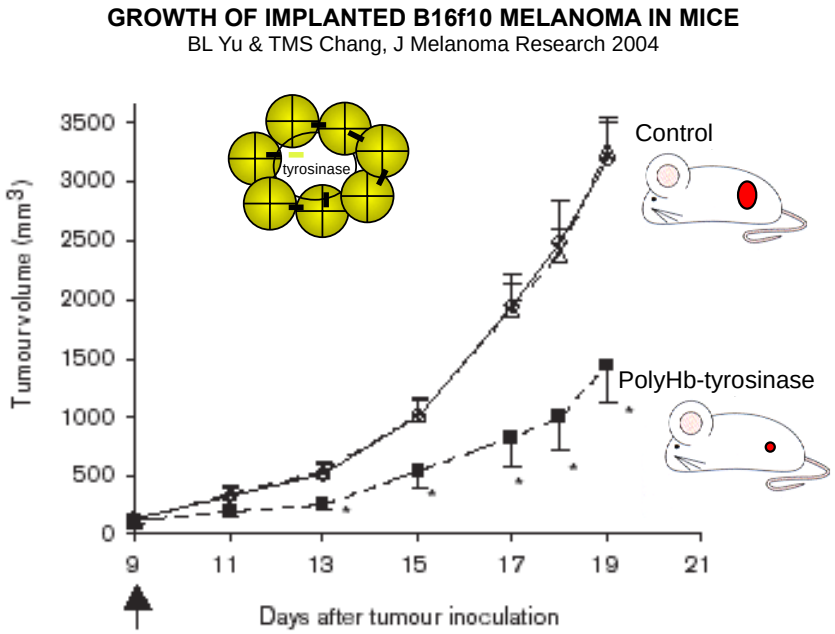

Figure 10. PolyHb can better perfused the microcirculation of tumours. This increases the low oxygen tension in tumour and thus increases their sensitivity to radiation and chemotherapy. PolyHb-tyrosinase combine this effect with the removal of tyrosine needed for the growth of melanoma. Effects of daily intravenous injection of PolyHb-tyrosinase on tumor growth of B16F10 melanoma in mice. (i) sham control: no intravenous injection; (ii) saline control: (iii) PolyHb-tyrosinase group. (With copyright permission from Chang 2007)

9. STEM CELLS FOR BLOOD SUBSTITUTES

There is much potential for the use of stem cells for the production different types of blood cells. This may be most useful for platelets and leucocytes since only small amounts are needed. Even then, platelets, unlike nanobiotechnological derived ones, has extremely short storage life. In the case of red blood cells, despite much research, it is still not possible to scale this up sufficiently for the large volume of rbc needed (Mazurier et al 2011). When scale up becomes a reality, this will be an important source of rbc for many clinical conditions. However, for other uses, (Chang 1964, 1965) these rbc will still have many of the same problems of rbc. These include:

- Even with refrigeration rec blood cells but still have a short storage time at 4C of less than 42 days. PolyHb can be stored in room temperature for more than 1 year. Freeze-dry powder of PolyHb and PolyHb-enzymes have even longer stability.
- Red blood cells cannot be freeze-dry into powder form. PolyHb. Conjugated Hb and PolyHb-enzymes in the freeze dry form are light and compact with ease of transport and storage for emergency, major disaster or war.
- Unlike red blood cells, HBOCs can better perfuse obstructed microcirculation as in stroke, heart attack, ischemic limbs, sickle cell anemia and other conditions. It can also better perfuse disturbed microcirculations as in tumour, hemorrhagic shock and other conditions.
- Unlike red blood cells, PolyHb-enzymes can be enhanced with higher enzyme levels than red blood cells to be more effective against severe ischemia-reperfusion injury, fatal elevation of tissue pCO2 and other conditions.
- Nanobiotechnology can combine Hb with other enzymes and other bioreactants for specially designed oxygen therapeutics

10 FUTURE PERSPECTIVES.

10.1. Blood substitutes

The first nanobiotechnological blood substitutes were reported in the 1960s (Chang, Science, 1964,1965, Bunn & Jandl, 1968). Most people thought that blood substitute was a simple matter that could be quickly developed when needed. Thus, blood substitute research was put aside and only the other areas of artificial cells were developed around the world. When AIDS came in 1989 there was no blood substitutes and many patients were infected with H.I.V. contaminated donor blood. It was only then that intense R&D on blood substitutes was belatedly carried out around the world. It was found out too late that blood substitute requires the same long-term research as in any other medical research for cancer and other diseases. Thus, after more than 20 years, only polyhemoglobin has been approved but only for South Africa and Russia. Much more research and development is still waiting to be carried out (Przybelski et al 1996, Tsuchida 1998, Klein 2000, Kobayashi et al 2005, Winslow 2006, Zuck 2006, Liu & Xiu 2008, Mozzarelli & Bettati 2011, Zapol 2011, Yang, Liu, Zheng 2013, Chang 2013, Kim & Greenburg 2014, Weiskopf 2014):

International progress up to now shows that it is possible to tailor-make blood substitutes ranging from simple to complex. It is urgent to have these ready without again waiting until it is too late. We need to analyze the specific indications for the different generations of blood substitutes. If a condition only needs an oxygen carrier, then there is no need to use a more complex one. On the other hand, it would be folly not to use a more complex one if indicated. We also need to intensify research on the many important ongoing research around the world These include: develop other novel approaches including novel crosslinkers; new sources of material from porcine, bovine, human cord rbc, recombinant, Arenicola marina; basic research on nitric oxide, oxidative stress, haptoglobin, rate of oxygen supply; safety and efficacy analysis and many other areas. The 2015 XV Int Symposium on Blood Substitute at Lund University (Professor Lief Bulow), Lund, Sweden http://isbs2015.lu.se , the 2017 XVI at McGill University (Professor TMS Chang), Montreal Canada www.medicine.mcgill.ca/artcell the 2019 XVII at Nara, Japan (Professor Sakai and Professor Yang) and the forthcoming 2021 XIII at Berlin, Germany(Professor Bumler) have been excellent opportunities for international exchanges.
Enormous amount of resources has been placed into basic research and developments on cancer, rare genetic diseases, molecular biology, organ failure and other areas. It is not reasonable to expect that for blood substitutes, we should be able to come out with a perfect blood substitute with little or no resources for academic and industrial research and development. Let's not wait for another crisis before we are again forced to do catch-up R & D.

10.2. Other areas:

This author predicted in his 1972 monograph on Artificial Cells (Chang 1972) that *"Artificial Cell is not a specific physical entity. It is an idea involving the preparation of artificial structures of cellular dimensions for possible replacement or supplement of deficient cell functions. It is clear that different approaches can be used to demonstrate this idea*". This prediction is already out of date, since the idea of artificial cells has progressed way beyond this 1972 prediction (Chang 2019).

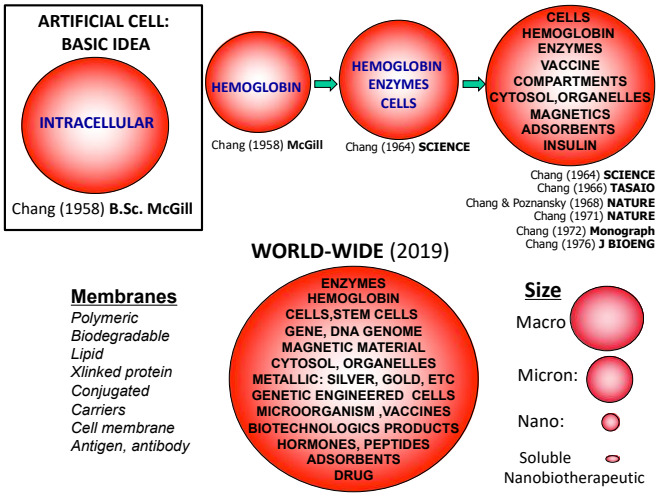

Fig. .10. Upper *(**from left to right**: Basic idea of artificial cells that led to different types of early artificial cells.* ***Lower:*** *Present status of artificial cells with unlimited variations in contents, membrane material and dimensions. From Chang 2019 with copyright permission.*

There are unlimited possibilities in variations for the artificial cell membranes and contents (Fig. 10). Artificial cells can now be of macro, micro, nano and molecular dimensions. Each of these has unlimited variations in configurations. Each configuration resulted in a new terminology that makes the field rather confusing to new comers (Fig. 11).

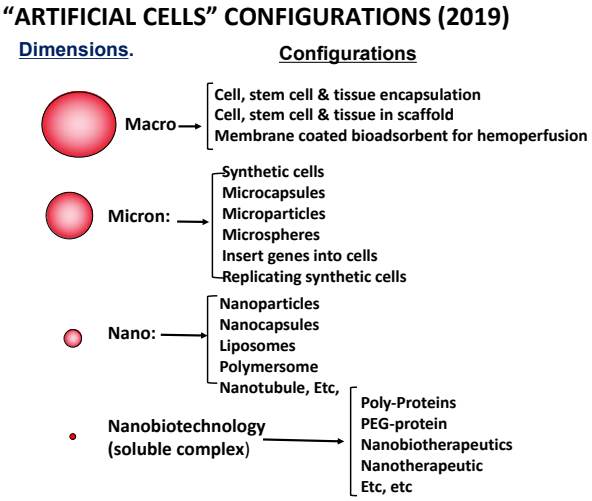

Figure 11: *Artificial Cell dimensions: macro, micro, nano and soluble nanobiotechnologic. Examples of variations in configurations with new terminologies for each extension. From Chang 2019 with copyright permission*

TABLE I

ARTIFICIAL CELLS: APPLICATIONS (2019)

Microdevice and nanodevice
Drug delivery:
Blood Substitutes and oxygen therapeutics
Biotherapeutics, Immunotherapeutics:
Enzyme and gene therapy:
Cell & Stem Cell Therapy:
Biotechnology & Nanobiotechnology
Nanomedicine
Regenerative medicine
Agriculture, Industry, Aquatic culture
Nanocomputers and nanorobatics
Nanosensors
Replicating synthetic cells etc
Other transformative possibilities

We have only touched the surface of the enormous potential of the extension, innovations and uses of artificial cells (Fig.10-11 and table I). More up to date details are available elsewhere (Chang 2019).

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http://www.medicine.mcgill.ca/artcell/2015bian&chang.pdf

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