5.1. Introduction

As discussed in the previous two chapters, investigators have successfully used the basic principles of nanobiotechnology-based blood substitutes to develop clinically useful polyHb and conjugated Hb (Chang, 1964, 1965, 1972a). Are we now ready to ask the next question? Is it possible to develop the more complete artificial red blood cells (Chang 1967, 1964) for clinical use in patients?

In nature, hemoglobin and complex enzyme systems are retained inside red blood cells (Fig. 5.1). In this way, they can carry out their functions: to transport oxygen and carbon dioxide, remove oxygen radicals and prevent hemoglobin from being oxidized into methemoglobin. It has been showed as far back as 1957 (Chang, 1957), that the contents of red blood cells can be enveloped in artificial red blood cells using ultrathin polymer membranes (Fig. 5.1). In this form, the hemoglobin and the enzyme systems normally present in red blood cells are retained inside the membranes. At the same time, oxygen and carbon dioxide can equilibrate rapidly across the membranes to interact with the enclosed materials (Chang, 1957, 1964). The membranes of the artificial red blood cells would not become fragile after prolonged in vitro storage. The artificial red blood cells could be transfused without cross-matching, since they do not have any blood group antigens. In order to develop this basic idea of a complete artificial red blood cell for practical applications, researchers from
Fig. 5.1. *Upper:* Composition of red blood cells and artificial red blood cell. *Lower left:* Red blood cells. *Lower right:* First artificial rbc’s of 1 micron or larger diameters; first lipid membrane nanodimension artificial rbc’s, first nano dieamension biodegradable polymeric membrane artificial rbc’s.
around the world have devoted a tremendous amount of effort to the research.

There are three steps in the development of a complete artificial rbc (Fig. 5.1), i.e. developing: 1) a micron dimension artificial red blood cell; 2) a submicron lipid membrane artificial red blood cell; 3) a nanodimension biodegradable membrane artificial rbc.

5.2. Micron Dimension Artificial RBCs with Ultrathin Polymeric Membrane

The first attempt at preparing an artificial red blood cell involved replacing the red blood cell membrane with an ultrathin polymeric membrane (Chang, 1957). This results in an artificial rbc with the same contents as those in a rbc, but with an artificial membrane instead of the red blood cell membrane.

Hemoglobin

The content of rbc, hemolysate, enveloped in a butyl benzoate coated collodion membrane artificial cell, contains a combination of oxyhemoglobin and methemoglobin. The oxyhemoglobin retains its ability to combine reversibly with oxygen (Fig 5.2) (Chang, 1957). Chang and Poznansky (1968a) showed that if additional catalase is enclosed along with the red blood cell hemolysate, there is a marked reduction in methemoglobin formation. In the case of nylon membrane artificial cells, the preparative procedure results in the formation of a much higher proportion of methemoglobin (Chang, 1964, 1965). Red cell hemolysate enclosed in silastic rubber artificial cells (Chang, 1966) remains unaltered and can combine reversibly with oxygen. A Japanese group has also supported these findings. They showed that hemoglobin microencapsulated within polymer membranes like polystyrene, ethyl cellulose and dextran stearate (Sekiguchi and Kondo, 1966) would combine reversibly with oxygen (Toyoda, 1966).

Carbonic anhydrase

Carbonic anhydrase, present at high concentration in rbc’s, plays an important role in the transport of carbon dioxide. Carbonic anhydrase
Fig. 5.2. Oxygen dissociation curve of collodion membrane artificial rbc’s (scanned from Chang, 1957).

of the red blood cell hemolysate enclosed in artificial cells with collodion, nylon, or cross-linked protein membranes, retains its activity (Fig. 5.3) (Chang, 1964, 1965).

The carbonic anhydrase activity was measured as the rate of fall of pH when carbon dioxide was bubbled through a buffered suspension. Artificial rbc’s and rbc’s both accelerate the rate of fall of pH (Fig. 5.3). Acetazolamide completely inhibited the catalytic activity of both rbc’s and artificial rbc’s with membranes of nylon, cross-linked protein, or collodion. Samples of buffered saline that had been in contact with their own volume of artificial rbc’s for 24 h showed no detectable enzyme activity, indicating that the enclosed carbonic anhydrase had not leaked out during this period. Carbonic anhydrase in artificial cells have about 75% of the activity of the enzyme in rbc’s. Thus,
Carbonic anhydrase activity measured as rate of fall of pH as carbon dioxide is bubbled into the respective solution (modified from Chang, 1964).

carbonic anhydrase in artificial rbc’s acts efficiently in catalyzing carbon dioxide.

**Catalase**

Catalase is another important component of mammalian rbc’s. We have analyzed the catalase activity of collodion membrane artificial cells containing hemolysate (Chang, 1967; Chang and Poznansky, 1968a). Both its natural substrate, hydrogen peroxide, and its chemical substrate, sodium perborate, have been used. Catalase enclosed in collodion membrane artificial cells acts efficiently *in vitro* and acts efficiently *in vivo* for enzyme replacement therapy in mice with a congenital defect in their red blood cell catalase (Chang and Poznansky, 1968a). This will be described in detail in the next chapter.

**Immunological studies**

Human ABO blood group antisera cause marked agglutination of both incompatible human rbc’s and heterogeneous rbc’s. On the other hand, these antisera do not cause any agglutination of artificial
rbc’s containing rbc hemolysate, even though the hemolysate has been obtained from incompatible human rbc’s or heterogeneous rbc’s (Chang, 1972a). Thus, an artificial red blood cell membrane does not appear to possess the blood group antigens normally present in biological cell membranes.

5.3. Intravenous Injection

_Polymeric membrane artificial RBCs 1 micron and larger_

The fate of intravenously injected nylon membrane artificial cells containing hemolysate has been studied (Chang, 1964, 1965, 1972a). A suspension of $5 \mu \pm 2.5 \mu$ diameter (mean $\pm$ standard deviation) nylon membrane artificial rbc’s was infused intravenously over a period of 1.5 min, and samples were obtained from the anesthetized cat during and following the infusion.

The arterial level rose steeply during infusion, but upon discontinuation of the infusion, the level fell rapidly (Fig. 5.4). Thus, most of the artificial cells successfully completed one or more circulations, after which they were removed rapidly from the bloodstream. Experiments using $^{51}$Cr-labeled nylon membrane artificial cells showed that most of them were removed by the lung, liver, or spleen. Collodion membrane artificial cells are also removed rapidly from the circulation. Thus, it appears that nylon and collodion membrane artificial cells smaller than rbc’s are rapidly removed from the circulation. During the infusion, there is a fall in arterial blood pressure and an increase in venous pressure and other signs compatible with pulmonary embolism. These artificial rbc’s do not have the deformability of rbc’s that allows the latter to pass through capillaries of smaller diameters.

Can artificial rbc’s survive longer if they are made much smaller in order that they can pass through the capillaries? Unfortunately, it has long been known that even submicron foreign particles are removed very rapidly from the circulation (Halpern _et al._, 1958). After all, this is part of the body’s defence against circulating microorganisms and other foreign particles. Thus, we looked into another factor, the surface
property of artificial cells (Chang, 1964, 1965, 1972a). Red blood cells and other formed elements of blood have a negative surface charge due to the presence of n-acetylneuraminic acid containing mucopolysaccharide on the red blood cell membrane (Cook et al., 1961). Danon and Marikovsky (1961) found that the younger rbc’s were about 30% more strongly charged than the older ones obtained from the same blood sample. All this information led us to look into the effects of surface properties on circulation time for rbc’s, foreign particles, and artificial rbc’s (Chang, 1965, 1972a).

**Neuraminic acid and circulation time of red blood cells**

We first studied the effects of removing neuraminic acid from rbc’s on their survival in the circulation (Chang 1965, 1972a; Chang and...
Experiments on the survival of autologous $^{51}$Cr-labeled rbc's were carried out using four dogs (Fig. 5.5). First, $^{51}$Cr-labeled rbc's obtained from each of the dogs were reinjected. They stayed in the circulation with the usual lifetime of about three to four months for dog rbc's. The second injection consisted of rbc's obtained from each of the dogs and then treated with neuraminidase to remove neuraminic acid. The procedure for the use of neuraminidase to remove neuraminic acid follows that of Cook et al. (1961). Removal of neuraminic acid resulted in the rapid removal of these rbc's from the circulation, with the level falling exponentially with a half-time of only about 2 h (Fig. 5.5). The third injection consisted of normal $^{51}$Cr-labeled rbc's obtained from each of the dog. The circulation time

**Fig. 5.5.** Circulation time of reinfused autologous dog rbc's, tagged with $^{51}$Cr. Data from four dogs: each point represents the radioactivity of one blood sample from a limb vein. In each case, the first and third tests were with normal rbc's from the same dog. The second test was with neuraminidase-treated rbc obtained previously from the same dog. Ordinates give percentage of injected label in circulation at time of sampling. Note rapid disappearance of neuraminidase-treated rbc's. (From Chang, 1965, 1972a.)
was not significantly different from that for the first injection. These results led to our suggestion that the survival of rbc’s may be related to the neuraminic acid present in the membranes (Chang, 1965, 1972a; Chang and MacIntosh, 1964).

**Effects of surface charge of polymeric particles on circulation time**

These results led to the investigation of a simpler model system (Chang, 1965, 1972a). Polystyrene latex particles (Dow Chemical Co.) with diameters of $2.05\mu \pm 0.018\mu$ (mean ± S.D.) were diluted 1:100 in saline. A similar suspension was prepared using polystyrene latex sulfonated for 30 sec by Ag$_2$SO$_4$ and H$_2$SO$_4$, and then washed with saline (Chang, 1965, 1972a). With the sulfonated polystyrene latex, the initial counts were close to the expected number. Then the level declined, first rapidly and then more slowly. In the case of the control polystyrene latex particles, there constituted only 10 to 15% of the injected particles in the circulation in the initial count $1/2$ min after injection. The level then fell much more steeply, with a half-time of about 15 to 30 sec. Further experiments showed that the sulfonated polystyrene latex particles were not trapped to any extent by the lung, but were removed by the reticuloendothelial system. The control polystyrene latex particles, on the other hand, were trapped fairly efficiently by the lung, and very efficiently by the reticuloendothelial system.

**Artificial rbc’s with sulfonated nylon membrane**

The above results led us to prepare artificial cells with sulfonated nylon membranes (Chang, 1965, 1972a); We used sulfonated diamine such as 4,4’-diamino-2,2’-diphenyldisulfonic acid (Chang, 1964, 1965, 1972a; Chang et al., 1966). Sulfonated nylon membrane artificial cells of varying surface negative charges can be prepared by adding different amounts of the sulfonated diamine (e.g. 3 mM/liter) to the alkaline 1,6-hexanendiamine solution, and then the standard procedure for preparation of nylon membrane artificial cells as described in Appendix II can be used. The reaction is shown in Fig. 5.6.
Sulfonated nylon membrane artificial cells prepared as described in the foregoing have a negative surface charge compared to that of rbc’s. Although sulfonated nylon artificial cells are also removed rapidly, they survived in the circulation significantly longer than nylon membrane artificial cells (Fig. 5.7). In addition, the sulfonated nylon membrane artificial cells are less liable to be trapped in the pulmonary circulation and the reticuloendothelial system compared with nylon membrane artificial rbc’s.

**Polysaccharide incorporation**

Since natural rbc’s and the endothelium of vessels are covered by mucopolysaccharide, further experiments were carried out to incorporate a complex containing a mucopolysaccharide (heparin) into the collodion membrane of artificial cells (Chang *et al.*, 1967; Chang, 1970). Preliminary results showed that while both types were removed rapidly from the circulation, those with a heparin-complexed membrane survived significantly longer.
Summary

The in vitro results showed that artificial rbc's with the required in vitro properties to substitute rbc's can be prepared. However, the single major problem is the extremely short circulation time of the artifical rbc's. We found that they have to be less than 1 micron in diameter to avoid being trapped in the lung capillaries (Chang, 1965, 1972a). Even then, they are removed by the reticuloendothelial system. We attempted to look at why the rbc's circulated for so long, by using neuraminidase to remove sialic acid from the red blood cell membrane. We showed that the removal of sialic acid resulted in the rapid removal of the rbc's from the circulation (Chang 1965, 1972a, Chang & MacIntosh, 1964a) (Fig. 5.5). This observation led us to prepare artificial rbc's with modified surface properties.
This included the use of other synthetic polymers and cross-linked protein membranes (Chang 1965, 1972a); membrane with negative surface charge and polysaccharide surface as sialic acid analogues (Chang et al., 1976, Chang 1970); and lipid-protein and lipid-polymer membranes (Chang 1969d, 1972a). Some of these improved the circulation time. However, the circulation time was still not sufficiently long for practical applications.

5.4. Submicron Lipid Membrane Artificial RBCs

We prepared larger lipid membrane artificial cells where the lipid is supported in the form of lipid-protein membrane and lipid-polymer membrane (Chang 1969d, 1972a). In 1980 Djordjevich and Miller reported that they could prepare submicron 0.2 micron-diameter artificial rbc’s using lipid membrane vesicles to encapsulate hemoglobin (Fig. 5.8). This increased the circulation time significantly, although it was still rather short. Many investigators have since carried out research to improve the preparation and the circulation time. Examples of the many investigators are Beissinger; Domokos; Farmer; Gaber; Hunt; Kobayashi; Miller; Phillips; Rudolph; Schmidt; Szeboni; Takahashi; Tsuchida; Usuuba, and many others. Improvements include modifications of surface properties such as surface charge, and the use of sialic acid analogues. The most successful approach to improve the circulation time is to incorporate polyethylene-glycol (PEG) into the lipid membrane artificial rbc’s (Fig. 5.8), resulting in a circulation half-time of more than 30 h (Philips et al., 1999).

Rudolph’s group has carried out a very extensive study that has contributed much to this area (Rudolph, 1994, 1995, Rudolph et al., 1997). Tsuchida’s group has for many years carried out research and development leading to commercial development and preclinical animal studies in preparation for clinical trials (Tsuchida, 1994, 1998; Tsuchida et al., 1988, 1992). In North America, lipid membrane artificial red blood cell’s are called lipid encapsulated hemoglobin (LEH). In Japan, they called hemoglobin vesicles (HbV).

Lipid encapsulated hemoglobin (LEH) is a very large area that will be best left for later book volumes of our book series “Regenerative Medicine, Artificial Cells and Nanomedicine” This way, each specialist
Fig. 5.8. Left: Bilayer lipid membrane that forms the membrane of lipid encapsulated hemoglobin (lipid membrane artificial rbc’s). Right: Incorporation of polyethylene-glycol (PEG) to the lipid membrane resulted in marked improvement in the circulation time of hemoglobin PEG-lipid vesicles (PEG-lipid membrane artificial rbc’s).

in a special area can present in detail his or her own specialized area. Furthermore, there will not be any copyright infringement by the author of this monograph. The following is a brief overview of LEH.

**Composition and preparation**

LEH lipid membrane consists of cholesterol and phospholipids. One of the aims is to increase the encapsulation efficiency of hemoglobin within a minimal amount of lipid. This can be done by using anionic lipids. Furthermore, inclusion of anionic lipid significantly increases the circulation time (Awasthi et al., 2004). However, some anionic liposomes may interact adversely *in vivo* with the complement (Szebeni, 1998). On the other hand, it is important to remember that the same type of lipid produced by different manufacturers may have different abilities in terms of activating the complement (Chang and Lister, 1990). This further supports the view that there is a need to use the C3a screening test for each lipid before its use in preparing LEH (Chang and Lister, 1990). The use of a new synthetic anionic lipid, 1,5-diplamitoyl-l-glutamate-N-succinic acid may solve the problem of complement activation (Sou et al., 2003).
Tsuchida’s group has been scaling up the production of PEG-LEH for preclinical and clinical studies. Briefly, they carried out pasteurization at 60°C for 10 h to achieve viral inactivation. The conversion of hemoglobin to carbonylhemoglobin (HbCO) increases the stability of hemoglobin during pasteurization. During pasteurization, all the other proteins including red blood cell enzymes are denatured and precipitated, resulting in a pure Hb solution. This also removes the red blood cell enzymes that prevent metHb formation, for instance methemoglobin reductase system and catalase. Thus, they tried to co-encapsulate the reductant-like glutathione of homocysteine and catalase with the purified Hb. They could also regulate the oxygen affinity by co-encapsulating allosteric effectors. After pasteurization, the Hb solution is passed through a series of ultrafiltrators to remove any virus, and then concentrated by ultrafiltration to 40 g/liter. After adjustment for electrolytes, this is then encapsulated into the PEG-LEH. HbCO is then coverted back to oxyHb by illuminating the LEH with a visible light under O2. The group was able to use the extrusion method to prepare PEG-LEH at a pH of 7.0–7.4. Their mixed lipids contained dipalmitoylphosphatidylcholine (DPPC) as the main component. Using a number of progressing steps of extrusion, they finally obtained PEG-LEH with a diameter of 250 ± 20 nm with a Hb/lipid ratio of 1.7–1.8.

The Hb inside PEG-LEH, like the red blood cell Hb, does not exert a colloid osmotic pressure. Thus, human serum albumin, hydroxyethyl starch or dextran has to be added to the suspending solution. They chose to use recombinant human albumin since there is no immunological problem and infective agents are absent. For purpose of storage, they use LEH with PEG on the surface to prevent aggregation and deoxygenate the hemoglobin to prevent metHb formation. The group reported that their PEG-LEH can be stored at 4°C and 23°C for 2 years. PEH-LEH stored at 40°C became unstable after one year.

**Methemoglobin**

Reducing agents and glucose cannot cross the LEH lipid membrane. Thus, reducing agents from the plasma cannot cross the PEG-Lipid
membrane to reduce the formation of methemoglobin. Furthermore, all the red blood cell enzymes are removed during the pasteurization procedure. Even if the methemoglobin reductase system can be added, it will require glucose as a substrate to recycle the cofactors needed in the enzymatic conversion of methemoglobin back to hemoglobin. As a result, hemoglobin in LEH is slowly converted to methemoglobin after infusion at body temperature. Increase in circulation time would not serve any useful purpose if the enclosed hemoglobin is converted to methemoglobin in long circulating PEG-LEH. As a result, many studies have been carried out especially by Tsuchida’s group to resolve this problem. As mentioned above, Tsuchida’s group added antioxidants like glutathione or homocysteine and catalase to hemoglobin before the latter is encapsulated into the PEG-LEH. To slow down methHb formation during storage, the LEH can be deoxygenated by perfusing it with nitrogen gas and storing it at 4°C.

5.5. LEH in Animal Studies

Circulation time after infusion

As discussed under polymeric membrane artificial rbc’s above, size is very important as far as circulation time is concerned. LEH larger that 200 nm are removed rapidly. Those under 200 nm are less likely to be removed by the reticuloendothelial system. However, those of about 60 nm are removed quickly by the liver, most likely through the fenestrated hepatic endothelium (Liu et al., 1992).

Thus, up to a point, a smaller size is important in maintaining LEH in the circulation. However, decreasing the size of LEH would also decrease the encapsulation efficiency of hemoglobin. In this regard, Philips et al’s (1999) study showing that PEG-lipid membrane artificial rbc’s can have a markedly increased circulation time is very important. The use of PEG-lipid membrane will allow for the use of larger LEH, with a better efficiency for encapsulating hemoglobin. Thus, a size range of 210–275 nm is possible with PEG-lipid membranes since these still maintain a good circulation time (Awasthi et al., 2003). Larger than this and the LEH would be removed rapidly. Inserting
PEG-linked phosphatidylethanolamines (PEG-PE) into the outer layer of LEH, will improve the encapsulation efficiency of hemoglobin and the circulation time (Awasthi et al., 2003). Thus, PEG-LEH infused into rats shows a circulation half-time of 30 h. Most of the LEH are removed by the reticuloendothelial system (RES), liver and spleen. Compared to humans, rats have a highly active RES; thus, it has been suggested that a $T_{1/2}$ of 12–20 h in rats is equivalent to 40–60 h in humans (Woodle et al., 1995).

Similar results were obtained with PEG-lipid membrane artificial rbc’s prepared by Tsuchida’s group (Saiki et al., 2001; Sou et al., 2005). Their study using radioisotope-labeling showed that the circulation half-life was 32 h in rats with a dose of 14 ml/kg (Sou et al., 2005). Gamma camera imaging showed that they were removed mainly by the liver, spleen and bone marrow. They were found in the phagosomes of the macrophages one day after infusion (Sakai et al., 2001), but cannot be observed after seven days.

Safety of lipid membrane artificial rbc’s

As mentioned earlier, Tsuchida’s group at Waseda University is developing their PEG-lipid membrane artificial cells for commercial scale up in preclinical animal studies towards clinical trial. They have collaborated with Kobayashi at Keio University to carry out detailed safety and efficacy studies in animal (Kobayashi et al., 1997, Izumi et al., 1997; Saikai et al., 1997, Saikai et al., 2004a, Saikai et al., 2004b, Saikai et al., 2004c, Saikai et al., 2004d; Yushizi et al., 2004). The following is a summary of their results. For copyright reasons, many of the excellent figures could not be included in this monograph, but are available in the referred papers.

In their study, HbV (LEH) were suspended in 5 g/dl human serum albumin. The resulting colloid osmotic pressure was 20 mmHg and the viscosity, 3 cP, both similar to whole blood. They studied the effects of daily repeated infusion of HbV by following serum biochemistry (Sakai et al., 2004a, Sakai et al., 2004b, Saikai et al., 2004d). Indicators of liver function like albumin, ALT, AST and LDH stayed within the normal ranges. Bilirubin and ferric ion remained at a low level. The
lipid concentration increased, but returned to its original level in 2 wk, indicating that the lipid component of the HbV were metabolized in the body. They found that infusion of these 250 nm diameter PEG-lipid membrane artificial cells did not result in any vasopressor effects. This lends further support to the hypothesis that vasopressor effects is due to single tetrameric Hb crossing the intercellular junction of the endothelial cell lining of blood vessels.

**Efficacy in exchange transfusion, hemorrhagic shock and hemodilution**

The group carried out exchange transfusion of 90% of the blood volume in rats and studied oxygen transport by inserting needle oxygen electrodes into their renal cortex and skeletal muscle. When albumin alone was used for the 90% exchange, there was a marked decrease in both the arterial blood pressure and renal cortical oxygen tension followed by the death of the rats. On the other hand, all the rats survived when the 90% exchange transfusion was carried out using their artificial lipid membrane artificial rbc's suspended in human serum albumin. Both the arterial blood pressure and renal cortical oxygen tension remained unchanged. They also studied the use of recombinant human serum albumin (rHSA) instead of human serum albumin to suspend the HbV. HbV suspended in this way was found to be safe and effective as priming volumes for 50% hemodilution in cardiopulmonary bypass. All the rats survived with continuing growth as shown by body weight gains. The group reported no irreversible changes in plasma biochemistry, hematology and histopathology.

They also studied the use of rat HbV for resuscitation in hemorrhagic shock in rats (Sakai et al., 2004c; Yoshizu et al., 2004). In anesthetized rats, removing 50% of the total blood volume resulted in hypotension and metabolic acidosis. Infusion of either HbV in rHSA or autologous rbc’s in rHSAA restored the blood pressure. However, rHSA alone with HbV did not restore the arterial blood pressure and some of the rats in this group died.
Summary

The single major obstacle to the practical realization of the original artificial rbc’s (Chang, 1957, 1964, 1972a) was the low circulation time. With extensive research by many groups, the use of PEG-lipid membrane to form 250 nm-diameter PEG-lipid membrane artificial rbc’s has increased the circulation time to a clinically useful level. This is an important and major step in the development towards a more complete artificial rbc for clinical use.

5.6. Nanodimension Biodegradable Polymeric Membrane RBCs

Introduction

Submicron PEG-lipid membrane artificial rbc’s as described above, have solved the major barrier of short circulation time. The lipid encapsulated hemoglobin (LEH) has potential uses in a number of clinical conditions. However, in some other clinical conditions one may have to look at the effect of the lipid membrane of the LEH especially regarding the following two aspects.

(1) The smaller the particle, the larger will be the total surface area in the same volume of suspension. Thus, a 1 ml suspension of 200 nm-diameter LEH would have more than 10 times the total surface area of a 1 ml suspension of rbc’s. Since both have a bilayer lipid membrane, the total amount of lipid would, at least be 10 times higher for the 1 ml suspension of 200 nm lipid membrane artificial rbc’s compared to rbc’s. A large amount of lipid can decrease the phagocytic function of the reticuloendothelial systems (RES). In traumatic hemorrhagic shock, the RES needs to be efficient in removing contaminating microorganisms. Furthermore, in ischemic reperfusion, lipid may induce lipid peroxidation. Therefore, we are carrying out a study to see if we can cut down as much as possible the amount of lipid needed for the membrane of nano artificial rbc’s.
Another problem is methemoglobin formation. For example, reducing agents present in the plasma of the circulating blood can reduce the rate of methb formation in polyHb or conjugated Hb. However, these reducing agents cannot cross the lipid membrane of LEH. Glucose that is needed for the methemoglobin reductase system inside red blood cell’s also cannot cross the lipid membrane of LEG. Thus, research to find a way to prevent metHb formation in long circulating LEH is an ongoing process. For biodegradable membrane nano artificial rbc’s, we can use membranes that are permeable to reducing agents and small molecules like glucose.

**Nanodimension biodegradable polymeric membrane artificial rbc’s**

Our aim is, therefore, to study how to prepare nanodimension artificial rbc’s with the following properties:

1. Contains little or no lipid in the membrane.
2. Persist in the circulation after infusion for a sufficiently long time.
3. Be stable in storage.
4. Remains stable after infusion for the duration of its function as a blood substitute — but it has to be biodegraded soon after the completion of its action in the body.
5. The membrane material and its degradation products have to be nontoxic.
6. In addition to hemoglobin, the nano artificial cells should contain important rbc enzymes like superoxide dismutase, catalase, carbonic anhydrase and methemoglobin reductase, as shown in Fig. 5.1.
7. The membrane should be permeable to reducing agents and/or glucose.

We have been using biodegradable polymer, e.g. polylactic acid, to prepare micron dimension artificial cells for the encapsulation of hemoglobin, enzymes and other biologically active material since 1976 (Chang, 1976a). With the availability of methods to prepare artificial cells of nanometer dimensions, we started to prepare
hemoglobin nanocapsules of less than 0.2 micron mean diameter using polylactic acid (PLA) membrane, PEG-PLA membrane and other biodegradable polymers (Chang & WPYu, 1992, 1997a, 1998, 1999, 2001; Chang et al., 2003a, 2003b; WPYu & Chang, 1994, 1996).

**Safety and reasons for selection of polylesters as the biodegradable polymer membrane**

Polymers for forming nano artificial rbc membrane have to be nontoxic and can be degraded in the body into nontoxic degradation products. Polysters like polylactic acid, polyglycolic acid and their copolymers polylactoglycolides finally degrade into lactic acid and glycolic acid which are normal human metabolites. The rate of degradation can be adjusted by variations in the molecular weight, particle size and ratios of the copolymer. Other potentially useful biodegradable polyesters include Poly(hydroxybutyric acid) and its copolymer with hydroxyvalerianic acid, polyorthoesters, polyanhydrides and others. Potential toxicity can come from residue monomers and chemicals used in polymerization to form nanocapsules. The solvent evaporation method (Chang, 1976a) for preparing polylactide membrane artificial cells and microspheres is an extension of the original method for preparing artificial rbc’s (Chang, 1957). This method obviates the need for monomers or chemical reagents and nontoxic solvents can be used.

Since 1982, there has been extensive clinical experience gained in the use of polylactic acid, polyglycolic acid and polylactoglycolides in humans as surgical sutures or implants with no adverse or toxic effects. The amounts used are many folds greater that needed for the ultrathin membrane of the nano artificial rbc’s. Polyglycolic acid (Dexon-S) suture has been used routinely in surgical practice since 1982 with no reported toxicities. Meadows et al. (1993) show that grafting polylactic acid (PLA) granules is as successful as using decalcified freeze-dried bone allograft. There is no reported toxicity in many other surgical implantations of polylactide or polyglycolide in amounts many folds greater than what we plan to use for nano artificial red blood cell membrane. The basic approach of preparing polylactic acid artificial
cells or microspheres (Chang, 1976a) for drug delivery has been used clinically. For example, Jaquel et al. (1995) gave repeated injections of Poly(DL-lactate-coglycolide) microspheres containing Octreotide in 100 acromegalic patients.

As mentioned above, long term experiences in humans show that the polyesters and their degradation products are safe. We also have long term experience in research on biodegradable polymer microencapsulation using polylactides, having initiated this area in 1976 (Chang, 1976a). Thus, we decided to look into the use of polylactic acid as the biodegradable membrane for nano artificial rbc’s. Details of the method of preparation will be described later.

5.7. Characterization of Nano Artificial RBCs

Electromicroscopic appearance

A Philips EM300 electron microscope, at 60 KV, was used. The sample was treated by standard negative staining. A typical electron micrograph for the biodegradable polymer hemoglobin nanocapsules prepared with d,l-PLA is shown in Fig. 5.9. They are spherical and homogeneous. Their diameter in the micrograph ranges from 40–120 nm, with a mean diameter of 80 nm. The membrane thickness is 5–15 nm.

Size distribution of polylactide membrane artificial rbc’s

The diameter and size distribution of the biodegradable hemoglobin are determined by using the Nicomp Size Analyzer (Model 370). The instrument operates by light scattering. The average particle size of the biodegradable nanocapsules containing hemoglobin is dependent on the formula used for preparation. A typical size determined using the Nicomp Sizer Analyzer is presented in Fig. 5.10. A unimodal distribution is obtained for all the samples. With different preparation processes and different polymers, the mean diameters of the biodegradable hemoglobin nanocapsules can be shown to be as low as 74 nanometers.
Fig. 5.9. Electromicroscopy of polylactide membrane nano artificial rbc’s. Mean diameter is 80 nanometer (from WPYu & Chang, 1996).

![Image](artificial_cells.png)

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<td>173</td>
<td>78.1</td>
<td>11.9</td>
</tr>
<tr>
<td>202</td>
<td>55.4</td>
<td>8.4</td>
</tr>
<tr>
<td>237</td>
<td>34.0</td>
<td>5.2</td>
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<td>278</td>
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<td>2.7</td>
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<td>8.2</td>
<td>1.3</td>
</tr>
<tr>
<td>382</td>
<td>3.3</td>
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</tr>
<tr>
<td>448</td>
<td>1.1</td>
<td>0.2</td>
</tr>
<tr>
<td>525</td>
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<tr>
<td>991</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Fig. 5.10. Size distribution of PLA nano artificial rbc’s determined using the Nicomp Size Analyzer (Model 370). An unimodal distribution is obtained. The mean diameter for this sample is about 100 nm. (From WPYu & Chang, 1996).

Other properties

The characteristics of the preparation containing 10.97 gm/dl of hemoglobin prepared with d,l-PLA is listed in Table 5.1. Nanocapsules can be prepared with up to 15 gm/dl hemoglobin concentration.
Table 5.1 Characteristics

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hemoglobin concentration</td>
<td>10.97 g/dl (up to 15 g/dl)</td>
</tr>
<tr>
<td>Polymer concentration</td>
<td>1.2 g/dl</td>
</tr>
<tr>
<td>Phospholipid concentration</td>
<td>0.6 g/dl</td>
</tr>
<tr>
<td>Specific gravity (22°C)</td>
<td>1.0047</td>
</tr>
<tr>
<td>Viscosity (37°C)</td>
<td>3.7 to 3.8 cp</td>
</tr>
</tbody>
</table>

Table 5.2 Steady Shear Viscosity (22°C, cp)

<table>
<thead>
<tr>
<th>Shear Rate (1/sec)</th>
<th>Viscosity of Preparation</th>
</tr>
</thead>
<tbody>
<tr>
<td>45</td>
<td>6.5</td>
</tr>
<tr>
<td>90</td>
<td>6.5</td>
</tr>
<tr>
<td>225</td>
<td>5.0</td>
</tr>
</tbody>
</table>

**Steady shear viscosity determination**

Steady shear viscosity of the suspension of the nano artificial rbc’s was measured with a Wells-Brookfield Syncro-Lectric Microviscometer (Model LVT) equipped with a 0.80° cone (Model CP-40). Shear rates were from 45 to 450 s\(^{-1}\) at 22°C. The steady shear viscosity of the biodegradable polymer hemoglobin nanocapsules is shown in Table 5.2.

5.8. Safety and Efficacy of Nano Artificial RBCs

**Amount and fate of PLA membrane**

By assuming that all the polymer used in the solvent evaporation method is incorporated into the nano artificial red blood cell membrane, the maximal amount of polymer per 500 ml of suspension of nano artificial rbc’s is shown in Fig. 5.11. The total amount of membrane material is much less that the total membrane material in LEH. Furthermore, the membrane material of PLA nano artificial rbc’s as shown in Fig. 5.11 is made up mostly of biodegradable polymer with a minimal amount of lipid. Since polymer is stronger than lipid and is also porous, much less membrane material is required.
Fig. 5.11. *Top:* Amount of membrane material in LEH and PLA nano rbc’s, *2nd from top:* Fate of polylactide membrane in PLA nano rbc’s compared to PLA metabolism. *3rd from top:* Hb concentration reported. *Bottom:* Oxygen dissociation curve of PLA nano rbc’s compared to SF Hb used in preparation.
Polylactic acid is degraded in the body into lactic acid and then to carbon dioxide (Fig. 5.11). These are all normal body metabolites. However, in hemorrhagic shock much lactic acid can be produced. Therefore, it is important to analyze how much lactic acid is produced in the degradation of PLA nano artificial rbc’s. Polylactide is degraded into lactic acid and then water and carbon dioxide (Fig. 5.11). For a 500 ml suspension of PLA nano artificial rbc’s, the total lactic acid produced is 83 mEq. This is far less than the normal resting body lactic acid production of 1000–1400 mEq/day (Fig. 5.11). The maximal body capacity to break down lactic acid is 7080 mEq/day. Thus, 83 mEq is equal to about 1 to 2% of this. Furthermore, the polylactic acid in the PLA nano artificial rbc’s is biodegraded over at least two days, and therefore, there is an even smaller amount released per day for each unit of PLA nano artificial rbc.

**Efficiency in nanoencapsulating hemoglobin**

At present, the hemoglobin nanoencapsulation efficiencies range from 13 to 29% of the starting quantities of hemoglobin, depending on the polymer used. A higher amount of hemoglobin is encapsulated with poly(D,L)-lactic acid. Nano artificial rbc’s prepared with poly(D,L)-lactic acid contained fewer defects in the membrane than that prepared using poly(L)-lactic acid. The ratio of hemoglobin: phospholipid : polymer for the preparation with hemoglobin of 10.97 gm/dl suspension is 15 : 1 : 2. Higher Hb concentration of up to 15 gm/dl suspension has also been prepared more recently (Fig. 5.11). This approaches the hemoglobin concentration in whole blood.

**Oxygen affinity, Hill coefficient and Bohr effect**

We use the TCS Hemoxanalyser (TCS Medical Products Co., U.S.A.) to measure oxygen affinity. The result for PLA artificial rbc’s with 10.97 gm/dl hemoglobin, is shown in Fig. 5.11. There is no significant difference between hemoglobin nano artificial rbc’s and the original bovine hemoglobin used for the preparation. 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. In the physiological pH range, oxygen affinity of PLA nano artificial rbc’s changes with pH (Fig. 5.12) and the Bohr effect is $-0.22$ to $-0.24$.

**Enzymes and multienzymes**

We have earlier carried out basic research on artificial cells containing multienzyme systems with cofactor recycling (Chang, 1987b). A number of enzymes normally present in rbc’s have been encapsulated within nano artificial rbc’s and they retain their activities (Chang *et al.*, 2003a, 2003b). For example, we have encapsulated the red blood cell methemoglobin reductase system into the nano artificial rbc’s (Chang *et al.*, 2003a, 2003b) and showed that this can convert methemoglobin to hemoglobin. For PLA nano artificial rbc’s, the membrane can be made permeable to glucose and other small molecules. This allows us to prepare nano artificial rbc’s containing
the methemoglobin reductase system to function as shown in Fig. 5.1. External glucose can diffuse into the nano artificial rbc’s. Products of the reaction can diffuse out and therefore do not accumulate in the nano artificial rbc’s to inhibit the reaction. Furthermore, reducing agents from the outside can enter the nano artificial cells to help in preventing metHb formation. These studies are discussed in more detail below.

**Nano artificial rbc’s containing metHb reductase system**

By increasing the circulation time, we can increase the *in vivo* functioning of the hemoglobin in the nano artificial rbc’s. However, with the increase in circulation in the body at 37°C, there would be a steady increase in methemoglobin. Oxidation of hemoglobin to methemoglobin inside rbc’s is prevented by the enzyme systems of the red blood cell (rbc).

PLA nano artificial rbc’s can contain all the enzymes of the red blood cell (Fig. 5.1). This is done by extracting from red blood cell all the enzymes and hemoglobin. In preparing nano artificial rbc’s, there is usually an increase in the metH level (Fig. 5.13). After this, when suspended in Ringer lactate at body temperature, metHb rose rapidly (Fig. 5.13). With the addition of 100 mg/dl glucose and 0.02 mM NADH, instead of increasing, metHb level decreased with time. This preliminary result is exciting because it shows that we only need to encapsulate fresh red blood cell contents with the normal amount of methemoglobin reductase system. In this way, 100 mg glucose (can be available as blood glucose) and 0.02 mM NADH in a suspending medium not only prevent metHb formation, but can convert preformed metHb back to Hb. Through further optimization of the NADH concentration, this can be increased further. Unlike NADH, the larger cofactor NADPH is not permeable across the nanocapsules. Since NADPH is not permeable, it can be enclosed inside the PLA nano artificial rbc’s for recycling. This would obviate the need to supply external cofactor and only a continuous supply of glucose from the circulating blood is needed.
Nanoencapsulation increases MetHb level. After this 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 decreases MetHb further.

**Nano artificial rbc's permeable to reducing factors**

There are reducing agents in the plasma that prevent methemoglobin formation. Examples include ascorbic acid and glutathione. These can cross the membrane of the PLA nano artificial rbc's. In our study, we find that ascorbic acid, glutathione or methylene blue can cross the membrane of PLA nano artificial rbc's. When nano artificial red blood cell is suspended in Ringer lactate with a 10 mM concentration of ascorbic acid, there is no increase in metHb as long as there is enough supply of ascorbic acid. (Fig. 5.13). Thus, it would appear that Hb in the nano artificial rbc’s could be exposed to reducing factors in the circulating plasma that prevent the formation of metHb. This is an additional and simpler means of preventing the oxidation of hemoglobin into methemoglobin.
**Enzymes and multienzymes in PLA nano artificial rbc’s**

Other red blood cell enzymes present in the red blood cell extract, like carbonic anhydrase, catalase and superoxide dismutase are also included in the nano artificial rbc’s (Fig. 5.1). In addition, higher concentrations of red blood cell enzymes like catalase and superoxide dismutase can be included. Other non-red blood cell enzymes can also be included.

### 5.9. Circulation Time of Nano Artificial RBCs

**Circulation half-life of PLA artificial rbc’s**

The *in vitro* results obtained so far appear promising and encourage us to continue to carried out detailed *in vivo* studies. We started with studying the circulation time. Circulation half-life of PLA nano artificial rbc’s is evaluated in anesthetized male rats. Each rat received 1/3 of its blood volume via intravenous top loading. PLA artificial red blood cell is removed very rapidly from the circulation.

As a result, we investigated different ways of changing the surface properties of the PLA nano artificial rbc’s as described below. This includes different ways of incorporating PEG into the PLA membrane.

**Different types of PEG-PLA copolymers for nano artificial rbc’s**

Figure 5.15 summarizes the results of the circulation time in rats for polyHb and different types of PLA and PEG-PLA nano artificial rbc’s (Chang *et al.*, 2003b). PLA nano artificial rbc’s are removed very rapidly from the circulation. PolyHb circulates with the same half-time as that normally obtained in rats. Depending on the method used, PEG-PLA nano artificial rbc’s (Fig. 5.14) have significantly higher circulation time than PolyHb. The details are as follows.

Anesthetized rats received a 30% blood volume toploading by intravenous infusion. Figure 5.15 shows that glutaraldehyde crosslinked polyhemoglobin (10 gm/dl) resulted in maximal non-red blood cell Hb concentration of 3.35 gm/dl, falling to half its maximal
concentration of 1.67 gm/dl in 14 h. From here on, PolyHb (17:1) is used as the basis for comparison with all nano artificial rbc preparations.

Four factors, individually or in combination, should be considered when preparing the different types of PEG-PLA nano artificial rbc’s. These are studied in regard to the maximal systemic Hb attained and the time of maintaining a systemic non-red blood cell Hb level equal to that of polyHb (17:1), 1.67 gm/dl. The four factors are as follows.

(i) Use of different degrees of polymerized Hb.
(ii) Effects of higher M.W. PLA.
(iii) Effects of concentrations of the PEG-PLA copolymer.
(iv) Crosslinking of the newly formed PEG-PLA Hb nanocapsules.

(1) Effects of molecular weight distribution of polyHb used in the PEG-PLA nano artificial rbc’s

The use of PolyHb (10:1) for PEG-PLA nano artificial rbc’s results in a maximal non-red blood cell Hb level of 3.05 ± 0.03 S.D. gm/dl.
Fig. 5.15. Comparison of the maximal systemic non-rbc hemoglobin reached after infusion of different preparations and the time to reached a given non-rbc hemoglobin level. The time for PolyHb-17 to reach a non-rbc hemoglobin level of 1.67 gm/dl is 14 hours in rats equivalent to 24 hours in human. The time for different types of nano artificial red blood cells to reach this non-RBC hemoglobin concentration is used to calculate the equivalent time for human (Chang et al., 2003b).

The non-red blood cell Hb falls to 1.67 gm/dl in 12.3 h (Fig. 5.15). Calculations based on body weight, blood volume, plasma volume and dilution factors show that the maximal non-red blood cell hemoglobin concentration for nano artificial rbc’s should have been 3.6 gm/dl. This seems to show that a significant part (about 16%) has been removed nearly immediately on infusion.

We then used a more refined method to improve the degree of polymerization to markedly reduce the amount of tetrameric hemoglobin (PolyHb 17:1). This was then used for preparing PEG-PLA nano artificial rbc’s. Two min after infusion, the maximal non-rbc Hb was $3.58 \pm 0.04$ S.D. gm/dl. This was significantly higher than the 3.05 gm/dl (S.D.= 0.04) above. This also approached the maximal possible initial non-red blood cell Hb concentration. Furthermore, it took 17.1 h in rats for the non-red blood cell Hb level to fall
to 1.67 gm/dl. The next step was to further improve this in the following step-wise incremental design until we reached a maximal concentration of $3.66 \pm 0.03$ S.D. gm/dl and 24.2 h to fall to the level of 1.67 gm/dl in rats.

(2) Effects of higher concentration of PEG-PLA copolymer combined with polyHb (17:1)

The same method as above with PolyHb (17:1) used, but with a 1.5 times higher PEG-PLA concentration. This resulted in a thicker membrane with better membrane stability, which in turn resulted in a further increase in circulation time (Fig. 5.15). Thus, in two min after infusion, the maximal non-red blood cell Hb was $3.60 \pm 0.01$ S.D. gm/dl. Furthermore, it took 20.0 h in rats for the non-red blood cell Hb level to fall to the level of 1.67 gm/dl. We also used twice the concentration of PEG-PLA polymer to further improve the stability of the membrane. However, the Hb nanocapsules formed this way tended to aggregate and therefore was not tested in animal studies.

(3) Effects of higher molecular weight PLA for the PEG-PLA copolymer

We looked at the use of a higher molecular weight PLA to increase the stability. For this we replaced the 5K PLA in (1) above with a 15K PLA to form the PEG-PLA copolymer. This significantly increases the circulation time of the preparation as compared to method (1). Thus, in two min after infusion, the maximal non-red blood cell Hb was: $3.57 \pm 0.05$ S.D. gm/dl. The slope of the disappearance was also much more gradual, but what is more important is that it took 21.2 h in rats for the non-red blood cell Hb level to fall to 1.67 gm/dl.

(4) Higher PEG-PLA concentration combined with higher molecular weight PLA

We next looked at combining the use of a higher molecular weight PLA (15K) as in (3) above with a 1.5 times higher concentration of
the polymer as in (2) above. This is a combination of the following 3 factors:

(a) Using polyhemoglobin with low percentage of crosslinked tetrameric hemoglobin.
(b) Using a 1.5 x concentration of the PLA-co-PEG copolymer.
(c) Using a higher molecular weight PLA (15K).

This resulted in a further significant improvement. In 2 min after infusion, the maximal non-red blood cell Hb was: 3.6458 ± 0.02 gm/dl. The slope of the disappearance was also much more gradual, and it took 23.3 h for the non-red blood cell Hb level to fall to the level of 1.67 gm/dl (Fig. 3).

(5) Effect of crosslinking the newly formed Hb nanocapsules

The method described in (1) above using polyHb (17:1) was modified by adding glutaraldehyde to the newly form PEG-PLA nano artificial cells. This approach has been used earlier to stabilize both the larger Hb microcapsules and the protein inside (Chang, 1971b). The polymerization was stopped by adding 2 M of lysine (at molar ratio of lysine/hemoglobin = 100:1) after 24 h. This approach also increased the circulation time to the same degree as when using 1.5 times concentration of the polymer in (2) above. Thus, in 2 min after infusion, the maximal non-red blood cell Hb was higher: 3.60 ± 0.01 S.D. gm/dl. The slope of the disappearance was also much more gradual, but what is more important is that it took 20.3 h in rats for the non-red blood cell Hb level to fall to 1.67 gm/dl (Fig. 5.15).

(6) Effects of the combination of all four factors to prepare Hb nanocapsules

Finally, we combined all the above four factors as follows:

(a) Using the polyhemoglobin (17:1) with a low percentage of single crosslinked tetrameric hemoglobin.
(b) Using a 1.5 x concentration of the PLA-co-PEG copolymer.
(c) Using a higher molecular weight PLA (15K).
(d) Crosslinking of the newly formed Hb nanocapsules with glutaraldehyde.

In 2 min after infusion, the maximal non-red blood cell Hb was higher at $3.6583 \pm 0.03$ S.D. gm/dl (Fig. 5.15). The slope of the disappearance was also gentler and it took 24.2 h in rats for the non-red blood cell Hb level to fall to 1.67 gm/dl.

**Analysis of results**

All the results obtained are summarized in Fig. 5.15 and Table 5.3.

After a 30% blood volume toploading using polyHb (10 gm/dl), the best polyHb-17 can only attain a maximal Hb concentration of 3.35 gm/dl. The best PEG-PLA artificial red blood cell’s, on the other hand, can reach a maximal Hb concentration of 3.60 gm/dl. This cannot be explained by polyHb having more colloid osmotic pressure than Hb inside the nanocapsules and resulting in some hemodilution. After all, in those PEG-PLA Hb nanocapsules that are removed more rapidly than PolyHb (17:1), e.g. nanocap-10-1-5K, the maximal Hb reached for nanocap-10-1-5K is lower than that for PolyHb-17. The more likely explanation is that for the best PEG-PLA Hb nanocapsules, they all remain in the circulation when the first samples are taken at 2 min after infusion. For nanocap-10-1-5K and PolyHb, a small fraction must have been removed within 2 min after infusion.

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Max Hb</th>
<th>Time to 1.67 g/dl (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PolyHb-10</td>
<td>3.10 gm/dl</td>
<td>10.4 (rats)</td>
</tr>
<tr>
<td>PolyHb-17</td>
<td>3.35 gm/dl</td>
<td>14.0 (rats)</td>
</tr>
<tr>
<td>Nanocap-10-1-5K</td>
<td>3.05 gm/dl</td>
<td>12.3 (rats)</td>
</tr>
<tr>
<td>Nanocap-17-1-5K</td>
<td>3.58 gm/dl</td>
<td>17.1 (rats)</td>
</tr>
<tr>
<td>Nanocap-17-1.5-5K</td>
<td>3.60 gm/dl</td>
<td>20.0 (rats)</td>
</tr>
<tr>
<td>Nanocap-17-1-5K-XL</td>
<td>3.60 gm/dl</td>
<td>20.3 (rats)</td>
</tr>
<tr>
<td>Nanocap-17-1.5-15K</td>
<td>3.57 gm/dl</td>
<td>21.2 (rats)</td>
</tr>
<tr>
<td>Nanocap-17-1.5-15K</td>
<td>3.65 gm/dl</td>
<td>23.3 (rats)</td>
</tr>
<tr>
<td>Nanocap-17-1.5-15K-XL</td>
<td>3.66 gm/dl</td>
<td>24.2 (rats)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Max Hb</th>
<th>Time to 1.67 g/dl (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

In humans, the maximal Hb concentration is achieved later, typically 30 min after infusion.
Relevance for clinical application in humans

After a 30% blood volume toploading, the best polyHb can maintain a systemic hemoglobin level of 1.67 gm/dl for 14 h in rats (equivalent to about 24 h in humans). In the case of the best PEG-PLA nano artificial rbc’s a similar toploading can maintain a much longer systemic Hb level, reaching 1.67 gm/dl after 24.2 h in rats (Fig. 5.15). If one uses the rat results in this study for polyHb of 14 h and its clinical equivalent of about 24 h, we might calculate and extrapolate this as follows. For the best PEG-PLA nano artificial rbc’s with the ability to maintain a systemic Hb level reaching 1.67 gm/dl after 24.2 h, it is likely equivalent to about 41.5 h in humans (Fig. 5.15). Compared to humans, rats have a highly active RES; thus, it has been suggested that a $T_{1/2}$ of 12–20 h in rats is equivalent to 40–60 h in human (Woodle et al., 1995). If this is the case, then the best type of PEG-PLA nano artificial rbc’s may have an equivalent circulation time in humans of more than 60 h. This is a significant and important increase that would allow longer function after infusion and thus decrease the need for donor blood and further increase the avoidance of donor blood. A further advantage of PEG-PLA nano artificial rbc’s containing polyHb instead of Hb is that even if PolyHb leaks slowly after infusion it would continue to act and, unlike tetrameric Hb, would not cause adverse effects. Indeed, PEG-PLA nano artificial cells could be useful carrier 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.

5.10. General

As discussed in Chapter 4, successes in nanobiotechnology-based polyHb and conjugated Hb stimulate research into the next generation systems. This is also the case in nanobiotechnology-based artificial rbc’s. Thus, research in micron dimension artificial rbc’s has led to the successful development of lipid encapsulated hemoglobin. This has encouraged us to look into the next step towards a further generation of nano artificial rbc’s in the form of PEG-PLA nano artificial rbc’s. We are only at the early stage of this research. In the meantime, it
is important to complete the development of the lipid encapsulated hemoglobin for clinical trials.

5.11. **Method of Preparation of Biodegradable Polymeric Nano Artificial RBCs**

*Organic phase*

Dissolve 100 mg (d.l)-polylactic acid (MW 25000) (Polysciences, Warrington, PA) in 8 ml acetone. Dissolve 50 mg hydrogenated soybean phosphatidylcholine (Avanti Polar Lipids, Alabaster, AL) in 4 ml ethanol with help of Ultrasonic water bath (very low power). The two solutions are mixed (added either one to another), and used as organic phase.

*Aqueous phase*

Take 0.04 ml of Tween 20, mix with 25 ml 15 g/dl hemoglobin.

*Preparation of PLA nano artificial red blood cells*

Slowly inject (8 ml/min). (The injection head is made with a 0.2 ml pipette tip). The organic phase integrates into the aqueous phase under magnetic stirring [with Therm-O-Swirl Stirrer (Precision Scientific Co., Chicago) setting to 6)], under 4°C. Nanoparticles are formed immediately, and the suspension is stirred continually for 15 min. The suspension prepared comes up to 37 ml.

The organic solvent is partly removed from the above suspension prepared by rotary evaporator under vacuum at 20°C for about 10 min. 33 ml of the suspension is obtained (i.e. 4 ml of organic solvent removed). The remaining suspension is mixed with 15 ml of 0.9% NaCl. Then the organic solvent and free hemoglobin are removed by ultrafiltration (by Amicon ZM 500,000 membrane, MW cut off 500,000). The suspension is repeatedly washed by 0.9% NaCl by ultrafiltration.

The detailed washing process is as follows:

a. The original 33 ml of Hb nanoparticles suspension plus 15 ml 0.9% NaCl, are concentrated to 10 ml.
b. 10 ml of suspension plus 40 ml 0.9% NaCl are concentrated to 6 ml.

c. 6 ml suspension plus 18 ml 0.9% NaCl are concentrated to 8 ml.

Preparation of PEG-PLA nano artificial red blood cells

The procedure for the preparation of the new PEG-PLA copolymer is as follows. 1/½g DL-PLA (M.W. 5000 or 15,000) and 0.75 g methoxypolyethylene glycol (M.W. 2000 or 5000) are dried under vacuum overnight. 5 ml acetone is added. The mixture is heated to 180°C for 2 h. under nitrogen. After adding 10 μl of stannous-2-ethylhexanoate, the mixture is heated at 180°C for another 3 h under nitrogen. This is soluble in acetone but not in water.

This PEG-PLA is used for the preparation of nano artificial cells. The procedure is similar to the procedure for the preparation of PLA nano artificial rbc’s described above, with the following change. 150 mg PEG-PLA copolymer is dissolved in 8 ml acetone. 50 mg hydrogenated soybean phosphatidylcholine is dissolved in 4 ml ethanol. The two solutions are mixed to form the organic phase.