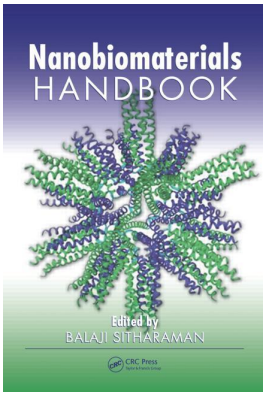

Nanobiomaterials Handbook

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Nanomaterials for Artificial Cells

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22.1 Introduction

The development of nanomaterials offers many promising solutions to problems facing the medical field. One possibility on the horizon is the development of artificial cells using nanomaterials. Engineered artificial cells can be used to replace dysfunctional cells in the human body and may be used in the future to treat anemia, renal failure, bone defects, and many other health problems. The biggest advantage in using nanomaterials in the fabrication of artificial cells is their small size, ranging from 1 to 100 nm in diameter (Liang et al. 2008). The small size allows a larger and more beneficial surface area to volume ratio and contributes to their unique physical and chemical properties. Biological cells have a very high functional density and contain DNA coding for thousands of different proteins to carry out certain functions. Engineering artificial cells with the same magnitude of functional density as biological cells is a significant challenge and has only recently become feasible with advances in nanotechnology.

In essence, an artificial cell or cells are systems that biomimic native cellular function to recapitulate either function and/or structure. This definition includes capsules or particles that biomimic a single or many biochemical functions of a cell of interest or the cell itself or encapsulations of cells to allow the cells to perform the function of choice. Artificial cell technology seeks to address the need for a more efficient temporary if not permanent replacement. In contrast to tissue engineering, the field of artificial cells is concerned with singular cells and recapitulations of functions, instead of whole complex tissues. The wide range of artificial cells can be glimpsed in the various applications that arose ranging from whole cell encapsulations of pancreatic islet cells and hepatocytes, liposome encapsulation of hemoglobin (Hb), and polymerized Hb. In the treatment of enzyme and single system defects, the application of whole cells may be detrimental, and replacing the enzyme or the single system may be more efficient as

is the case with artificial red blood cells. The application of nanobiomaterials is necessary to both better biomimic cellular systems and construct a more efficient system than nature itself.

In this chapter, we first briefly discuss the application of nanomaterials for artificial cells in general and then discuss the application of nanomaterials for artificial blood cells in details.

22.2 Development of Artificial Cells Using Nanomaterials

An important aspect for developing artificial cells is the cell membrane. The membrane serves as an interface for intercellular communication, and so it therefore plays a large role in drug delivery. The surface properties of nanoparticles must be considered while developing drug delivery systems and artificial tissues. Geometry of the membrane should also be considered when constructing artificial cells. One study comparing concave and convex membrane structures of poly(dimethylsiloxane) (PDMS) found that concave structures suppress cell adhesion and proliferation (Sun et al. 2008). Furukawa et al. (2007) were able to grow a lipid bilayer on a silicon wafer and glass because of its self-spreading properties at a solid–liquid interface (Liu et al. 2008). Supported lipid bilayers have been grown from a lipid molecule on a hydrophilic surface with nanostructures (Isenberg et al. 2008). Chen et al. were able to assemble a sandwiched, layer-by-layer lipid bilayer using 1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine (DMPE) on a polyelectrolyte multilayer (PEM) film (Romberg et al. 2007). In another study, 2-methacryloyloxyethyl phosphorylcholine (MPC) polymers with an attached phosphorylcholine side chain were used to mimic the phospholipids in cell membranes. This proved to be an effective way of establishing a membrane-like interface between artificial and biological materials (Ishihara et al. 2008).

The machinery needed in an artificial cell depends on the function that the cell needs to carry out. Progress has been made toward constructing artificial organelles, which is a stepping stone in the development of a complete artificial cell with similar functionality to a biological cell. In one study, a functional artificial Golgi apparatus was constructed using digital microfluidics, recombinant enzyme technology, and magnetic nanoparticles. The Golgi was able to modify glycosaminoglycans immobilized onto magnetic nanoparticles (Lin et al. 2007).

When developing artificial cells using nanomaterials, different techniques must be considered. There are two primary techniques for building nanomaterials: the "bottom-up" approach and the "top-down" approach. "Bottom-up" assembly requires the self-assembling of molecules, molecular manipulation, and molecular binding (Kabanov 2006). Bottom-up approaches rely heavily on the self-assembly of molecules from molecular recognition. The technique requires the assembly of nanomaterials through molecule by molecule or even atom by atom (Zhang 2003). One example of this is base-pairing in the synthesis of DNA. Bottom-up construction of nanomaterials is advantageous because of the opportunity to introduce functionality and precision when engineering nanomaterials (Furukawa et al. 2007).

The top-down approach involves fabricating small nanomaterials using larger materials to direct the assembly (Furukawa et al. 2007). Biomaterials are developed by stripping down a complex entity into its component parts (Zhang 2003). The advantage to using the top-down approach is the ability to organize nanomaterials in hierarchal patterns on a larger scale (Furukawa et al. 2007). Although bottom-up techniques have their advantages, it can be difficult to control a self-assembling system to engineer a specific desired structure (Vasita and Katti 2006). Therefore, integration of the bottom-up and top-down approaches is a possible solution and has been done in some studies (Vasita and Katti 2006). In one experiment involving DNA, the bottom-up and top-down techniques were integrated to create an array of DNA nanotubes (Furukawa et al. 2007). In another experiment, lithographic templates were used as a top-down approach to control the bottom-up self-assembly of block copolymers (Vasita and Katti 2006).

Self-assembling nanoparticles as a bottom-up approach has become a very common method in developing nanomaterials for artificial cells. Research involving the self-assembly of designed artificial peptides and peptidomimetrics into nanofibers has shown promise in developing a new class of soft-materials. These materials have the potential to be used in tissue engineering and biomineralization (Higashi and Koga 2008). A self-assembly method involving single-walled carbon nanotubes (SWNTs)

alternately packed β-1,3-glucans into the structure as building blocks. This method allowed for a hierarchal "superstructure" to be manufactured and may have future applications (Numata et al. 2008).

For successful surface modification of nanoparticles to take place, the membranes must be subject to broad adsorption of a variety of proteins and functional groups. On the other hand, the surface must be biocompatible. This is especially important in the bloodstream. Nanomaterials have to be particularly stable with regard to blood plasma proteins and platelets to prevent clotting. Various polymer coatings on synthesized nanoparticles have been utilized to mimic a biological membrane while maintaining blood compatibility (Ishihara et al. 1998). To reduce adhesion of plasma proteins, there has been development of polymers containing a 2-methacryloyloxyethyl phosphocholine moiety, or MPC polymers (Webb et al. 1995). More examples of polymer coatings that are functional include diacetylenic phospholipids, phosphatidylcholines, and phosphorylcholine groups (Webb et al. 1995). Surface modifications of nanoparticles for artificial cells not only serve to improve drug delivery, but also reduce the adverse effects of the agglutination of blood platelets and plasma proteins with nanomaterials.

There have been many different types of artificial cells developed as a result of the emergence of nanotechnology. Yi et al. (2009) were able to engineer an artificial amoeba that was capable of propelling itself by nanoparticle-triggered actin polymerization. Nickel and gold nanoparticles with attached Listeria monocytogenes transmembrane protein ActA were utilized along with actin, actin-binding proteins, and ATP and encapsulated in a lipid vesicle. The system served as an artificial cell with actin polymerization within the cell acting as a functional cytoskeleton (Yi et al. 2009).

One exciting development in research of artificial cells is that of the capsosome. A capsosome is a polymer capsule containing multiple liposomes (Chandrawati et al. 2009). In one study, capsosomes were fabricated by enveloping liposomes between a cholesterol-modified poly(L-lysine) (PLLc) precursor layer and a poly(methacrylic acid)-co-(cholesteryl methacrylate) (PMAc) capping layer (Chandrawati et al. 2009). Capsosomes can serve as microreactors to cause a series of reactions and release desired products to the surrounding environment (Chandrawati et al. 2009). A similar experiment utilized a multilayer film assembly of the polyelectrolytes poly(styrene sulfonate) (PSS) and poly(allylamine hydrochloride) (PAH) with liposomes (Städler et al. 2009). This technique yielded stable capsosomes, and shows the effectiveness of using polyelectrolyte capsules in capsosome formation. The development of capsosomes shows that the possibility of fabricating a biologically similar artificial cell or artificial organelles is not far off the horizon, and can lead to many new exciting possibilities in biotechnology.

Another development in the realm of artificial cells is the use of silica-coated beads for in vitro protein synthesis. Lim et al. (2009) found that the beads can encapsulate transcriptional and translational machinery for the synthesis of functional proteins. It was also confirmed that permeation of the proteins through the particle membrane was taking place. Silica-coated beads showed an increase in synthesized protein activity by fivefold and threefold over bare and chitosan-coated beads, respectively (Lim et al. 2009). The development of this type of nanoparticle can lead to the development of artificial cells through the parallel synthesis of varying functional proteins in designed combinations.

There are a wide variety of different types of nanomaterials being developed for use in artificial cells. Development of individual cell components such as the cell membrane, cytoskeleton, surface properties, and organelles is heavily underway. The possibilities for nanomaterials and artificial cells in the medical field are endless.

22.3 Nanobiomaterials for Artificial Red Blood Cells

One major finding in the development of artificial cells is their possible use as oxygen carriers in the bloodstream for the treatment of anemia. Blood substitutes, unlike blood itself, serve solely to carry oxygen and carbon dioxide throughout the body. A lot of the research being done focuses on hemoglobin-based products, which include PEG modified liposome-encapsulated hemoglobin, nanoparticle and polymersome encapsulated hemoglobin, and polymerized hemoglobin solutions (Sarkar 2008). One example of an oxygen carrier encapsulates a solution of concentrated hemoglobin and is called

a hemoglobin vesicle (HbV). HbV was found to have oxygen-carrying capacity comparable to that of normal red blood cells (Bucci 2009). The fabrication of artificial red blood cells in the form of HbV offers promising opportunities in a clinical setting.

22.3.1 Artificial Cells as Blood Substitute

Blood serves as the first model for artificial tissue, since the individual cell of blood can be easily separated from the whole tissue and its individual function is the same. Within blood, the need for a practical RBC substitute is quite prevalent. In brief, the limited donor pool (Riley et al. 2007) and even smaller number of donations (Davy 2004), as well as the biological complications such as infections (Spies 2004) and antigen matching (Hill 2001) complicate finding safe and sufficient blood donation for patients. To address this clinical issue, Dr. T.M.S. Chang devised the first artificial cell, microcapsules of nylon containing hemoglobin (Hb). The idea was for the encapsulation of the macromolecule to allow function while allowing small molecules to pass into and out of the system, thereby allowing oxygen exchange. The idea led to a field of study pertaining to hemoglobin and hemoglobin encapsulated systems or hemoglobin-based oxygen carriers (HbOCs).

In the development of the first HbOCs, goal was to increase oxygenation of tissues comparable to the addition of pRBCs and scientists knew that Hb was the molecule primarily involved. The rationale was to infuse a patient with the protein that was responsible for oxygen exchange in tissues. Because the drive was to avoid usage of whole cells, encapsulation of RBCs was not considered a viable option. Thus, the first generations of HbOC products were nanoparticles of single bovine or human Hb proteins (Garby and Noyes 1959; Brunn 1972) and eventually intra- (Chatterjee et al. 1986) and intermolecularly (Gould and Moss 1996) cross-linked Hb particles. The progressive shift from stroma-free Hb to modified Hb and eventually polymerized Hb was driven by in vivo toxicity and optimization of circulation half-life. The first generation of blood substitute products faced renal toxicity due to tetramer dissociation into dimers and the subsequent release of iron ions and also had a short in vivo circulation half-life of 10–30 min due to size allowing easy glomerular filtration (Bunn and Jandl 1969) and reticuloendothelial clearance (Bunn 1972; Hersheko 1975).

To mitigate the problem of a short circulation half-life, many approaches were done in cross-linking the tetramers together with glutaraldehyde (Chang 1971) or other bifunctional cross-linking agents such as bis(N-maleimidomethyl) ether (BME) (Wedekind et al. 1985) and bis(3,5-dibromosalicyl) fumarate (DBBF) (Walder et al. 1979; Sloan et al. 1999). Through stabilization of the tetramer, the circulation half-life was tripled, but oxygen affinity of the Hb particles was too high and thus did not provide adequate oxygenation. The next generation of products were polymerized Hb and polymer conjugated Hb to allow for greater molecular weights and size. To lower oxygen affinity, various compounds such as pyridoxal phosphate were cross-linked to Hb (Greenberg et al. 1979; Seghal et al. 1981). The other approach was to use bovine Hb that naturally has a lower affinity for oxygen and does not require cofactors (Stonwell et al. 2001). All the artificial RBC products failed at various phases in clinical trials for humans to date.

An alternative approach was taken by chemists who formed fluorinated hydrocarbons or perflourocarbons (PFCs). PFCs have a linear oxygen affinity curve and require more to equate to Hb's oxygen carrying capacity (Riess 2006). PFCs avoided most of the complications due to Hb administration but were fraught with others such as platelet inactivation and increased leukocyte counts, and long-term storage in tissues required emulsifying agents (Lane 1995). An example of a major product that stemmed from the research was Flousol (Tremper 1983). PFCs were never approved for human use.

The next rational step was taken of encapsulating Hb into vesicles that biomimic a cell—liposomes and micelles (Shi et al. 2009). The two approaches resulted in similar complications of lower diffusion of small molecules to and from the system as well as low encapsulation efficiencies and high conversion rate into methemoglobin, the inactivated form of the Hb (Sakai et al. 2004). Liposome or micelle based encapsulation systems failed to be an adequate RBC replacement as of today.

The final step was to create enzyme bags with the functions of a RBC through encapsulation of proteins in polymer networks. This was first demonstrated by Chang et al. (1971) by including Hb as well

as superoxide dismutase (SOD) to increase circulation time, reduce oxygen affinity, and allow for tissue oxygenation. Through the work by Zhao et al. (2007) the need for nanoparticles was elucidated; more specifically, the diameter of particles around 100 nm had the longest circulation half-life. Another major advantage of nanoscale HbOCs is the ability to pass through occlusions that commonly occur in trauma and high cholesterol patients allowing for lower chance of infarction. The need for nanobiomaterials is imperative into the further development of the field of artificial RBCs and artificial cells.

22.3.2 Characterization of Artificial Red Blood Cells

There are several desirable properties when it comes to designing an ideal HBOC. Theoretically, the HBOC would solve the problems associated with traditional allogenic blood transfusions and be able to provide an urgent need of oxygen delivery to tissues. Therefore, the ideal HBOC should have adequate delivery of oxygen to all tissues, not transmit infectious diseases, be universally compatible without having to conduct lengthy cross-matching and typing, be easy to administer, and be readily available and at reasonable costs. Many of these properties are based on the physical and chemical characteristics of these artificial cells, which can be tailored to design the ideal blood substitute.

Particle size, surface charge, and surface hydrophobicity are all critical parameters that undermine the overall performance of a HBOC when is it clinically administered (Awasthi et al. 2003). Several investigations have been conducted in order to find the "ideal" size requirements for nanoparticles developed as oxygen carrying artificial cells. Theoretically, the nanoparticles must be able to circulate freely through even the smallest of capillaries, which can be as small as 4–7 μm in diameter, and so should be smaller than 4 μm in order to avoid embolism. However, nanoparticles above approximately 200 nm will be removed by the spleen through mechanical filtration and consequently accumulate in the spleen (Kissel and Roser 1991; Moghimi et al. 1993). On the other hand, nanoparticles with diameters below approximately 70 nm will be removed by the liver due to the possible penetration of such small particles through fenestrae in the endothelial lining of the liver, hence causing an increased accumulation in the liver (Litzinger et al. 1994). As a result, it has been suggested that the 70–200 nm range is optimal for intravenous delivery and circulation (Zhao et al. 2007).

Surface modifications that increase hydrophilicity, to some extent, can prolong the residence of nanoparticles in blood by evading rapid elimination from systemic circulation, a process usually performed by monocytes and cells of the mononuclear phagocyte system (MPS) (Avgoustakis et al. 2003). In general, a higher protein adsorbability of hydrophobic relative to hydrophilic surfaces has been related to the uptake and rapid removal of hydrophobic particles by phagocytosis (Illum and Davis 1986). Thus, coating the nanoparticle surface with a hydrophilic polymer such as poly(ethylene glycol) (PEG), either through covalent attachment or physical adsorption to the surface, has been illustrated to decrease uptake by the MPS (Li et al. 2001). Because PEG prevents interactions with other biological components in vivo, HBOCs modified with PEG have longer circulation times and are non-thrombogenic (Gref et al. 1995).

Many attempts have been made to investigate the effects of surface charge on the distribution of the nanoparticles in circulation, but the outcomes are contradicting. In one study, negatively charged nanoparticles, when compared with neutral and positively charged nanoparticles, were shown to facilitate clearance of the nanoparticles from blood circulation (Stolnik et al. 1995). Conversely, Gbadamosi et al. (2002) demonstrated that a smaller negative charge decreased uptake of the nanoparticles and Yamaoka et al. (1995) reported that the introduction of negative surface charges to dextran derivatives prolonged its half-life in circulation. Although these results suggest that the surface charge of nanoparticles is important in determining blood circulation time, much research needs to be conducted in order to establish the effect of the surface charge on nanoparticles used as oxygen carriers.

Oxygen affinity is measured as the partial pressure of oxygen at which hemoglobin is 50% saturated with oxygen, commonly denoted as p50. Hemoglobin (Hb) is a tetrameric structure ($\alpha_2\beta_2$) that has two distinct conformations of the subunits (oxy and deoxy). Hb's cooperative binding to oxygen results in the sigmoidal shape of the oxygen-hemoglobin binding curve. Briefly, if one oxygen molecule binds to a

heme group, Hb changes its conformation so that the binding of more oxygen molecules to the remaining heme groups is facilitated and a similar action is seen if a heme group releases its oxygen molecule (Awasthi 2005). The normal p50 of human Hb in red blood cells is approximately 28 mm Hg, whereas Hb itself has a p50 of about 10 mm Hg (Winslow et al. 1977). Generally, a high oxygen affinity is associated with a low p50 and a low oxygen affinity is associated with a high p50.

The oxygen affinity of Hb is influenced by many factors such as temperature, pH, and carbon dioxide levels. Within the red blood cell, various salts also affect the oxygen affinity such as chloride ions, ATP, and 2,3-diphosphoglycerate (2,3-DPG) (Kobayashi et al. 2004). In red blood cells, 2,3-DPG decreases the oxygen affinity by cross-linking the β chains of the tetramer and allows the oxygen molecules to be released. Soluble human Hb has an increased affinity for oxygen as a result of insufficient 2,3-DPG in the plasma (Arnone 1972). Such high affinity Hb would not function well as an oxygen delivering substance since the amount of oxygen released is highly decreased. A simple solution to this problem is the use of bovine Hb. The oxygen affinity of bovine Hb is not 2,3-DPG dependent but rather chloride ion dependent and there is a sufficient amount of chloride ions present in the human plasma (Franticelli et al. 1984). Additionally, chemical modifications of Hb by cross-linking, polymerization, or polymer linking have also shown significant improvements in the p50 of Hb.

Ideally, these artificial cells should have a p50 close to that of the red blood cells. Because these HBOCs are designed for use in emergencies and life-threatening issues, the optimum value of oxygen affinity depends on the exact physiological conditions under which the HBOC is used. Recent findings have supported the use of HBOCs with a low p50 in cases of severe blood loss. It has been demonstrated that under normoxia or mild hypoxia, a high p50 HBOC may be beneficial while in severe hypoxia, a low p50 HBOC may be better (Kavdia et al. 2002; Shirasawa et al. 2003). Overall, oxygen affinity is a property that needs to be highly controlled when designing a HBOC as it is critical in the loading and unloading of oxygen within the human body.

A significant challenge in the development of HBOCs is faced due to their effect on the vascular tone of blood vessels; typically hypertension was observed in studies with soluble Hb. Unlike Hb in red blood cells, soluble Hb (or tetrameric Hb) can be readily extravasated into the endothelial tissue where it can rapidly bind to and remove nitric oxide (NO). NO, also referred to as endothelial-derived relaxing factor, plays an important role in controlling smooth muscle relaxation of the blood vessels (Schultz et al. 1993). When oxygenated Hb (HbO_2) reacts with NO, NO is oxidized to nitrate (NO^{3-}) and HbO_2 is reduced to metHb. This results in significant vasoconstriction of the blood vessels and a consequent increase in blood pressure (Olson et al. 2004).

Although NO scavenging is an important contributor to the increased vasoconstriction, it is not the only rationalization. Several alternative theories have been developed to explain this phenomenon, which includes too much delivery of oxygen and the oxidation of Hb, which can result in heme loss and free radical formation. Increased oxygen delivery to arterioles is followed by an autoregulatory constriction of the arterioles and the capillary beds as a result of what is perceived to be an excess of oxygen delivery (Sanders et al. 1996). The oxidation of Hb can result in the formation of products that induces endothelial stress causing vasoconstriction. One way of countering the extravasation of Hb is to increase the molecular size by PEGylation or by polymerization as such molecules will not be small enough to pass through the endothelial tissue.

These physical and chemical characteristics can be adjusted and fine tuned to prepare artificial red blood cells with the desired properties, a critical advantage when designing artificial cells to be used in specific situations.

22.3.3 Nanobiomaterials for Artificial Red Blood Cells

22.3.3.1 Proteins

As mentioned earlier, the first generation of artificial RBCs were HbOCs that was composed of entirely Hb or protein with modifications. Proteins of nanometric dimensions can serve as the platform for

artificial cells (Baudin-Creuza et al. 2008). The first approach was to inject purified Hb or stroma-free Hb directly into animals and patient in place of RBCs. The idea of polymerizing Hb was extended to prolonging the circulation half-life and reducing renal toxicity of tetramer dissociation through cross-linking 4–5 Hb molecules with glutaraldehyde (GTA) and other bifunctional groups. Concurrently, Hb conjugated to polymers such as polyamide, dextran, and PEG were also done for the same effect of increasing circulation half-life. The origin of Hb has also changed from allogenic human Hb (Yamaguchi et al. 2009) to bovine Hb (Mullon et al. 2000) and eventually to recombinant Hb (Fronticelli and Koehler 2007). Few products have progressed into clinical trials. The first generation of products is exemplified by DCLHb or HemAssist™ by Baxter. HemAssist is a diaspirin cross-linked Hb system. The Hb was obtained from expired human pRBCs (Lamy et al. 2000).

PolyHeme™, product of Northfield Laboratories, is composed of human hemoglobin form expired pRBCs that have been pyroxylated and polymerized with GTA. The end concentration of Hb in the suspension is about 10 g/dL. PolyHeme has a P_{50} of 20–22 mm Hg, a little lower than intra-RBC Hb's 26 mm Hg. A single molecule has a molecular weight of 150 kDa and results in double the viscosity of saline solutions (Gould et al. 1995; Dubick et al. 2004). PolyHeme was shown to reduce mortality in patients with severe acute anemia when compared to patients rejecting pRBC transfusions.

Hemopure™ or HBOC-201, a product of Biopure, is a solution of purified bovine Hb with a final Hb concentration of 13 g/dL and a P_{50} of 36 mm Hg. It has a circulation half-life of about 19 h and can be stored up for over 3 years. Phase III clinical trials of HBOC-201 showed adverse side effects in 93% of patients including a rise in blood pressure and thus HBOC-201 was not allowed to enter phase IV in the United States (Wilson et al. 2007). It was, however, approved for adult use in South Africa and reports of cardiac arrest after usage have been published (Stefan et al. 2007). It is safe to note that the patient was a child and not an adult. Another product resulting from HBOC-201, Oxyglobin™, HBOC-301, is a GTA polymerized HbOC approved by the FDA for veterinary use (Jahr et al. 2007). It was shown by Buehler et al. (2005) to have a P_{50} of around 38.4 mm Hg compared to bHb, which had a P_{50} of around 27.2 mm Hg. The stability and viability of the GTA polymerized Hb was demonstrated in animal models.

Another approach toward prolonging of in vivo circulation half-life and lower oxygen affinity for both human and bovine stroma-free Hb was to cross-link the Hb with polymer chains; the system is also referred to as conjugated Hb in literature. The polymer chains employed with both synthetic and naturally occurring. A common approach was to cross-link PEG to polymerized Hb (Iwashita et al. 1988; Shorr et al. 1996). PEG is a good candidate for increasing molecular weight because it is a synthetic polymer that can be obtained at desired molecular weights, is easily water soluble, and is non-thrombogenic (Watanable et al. 2004; George et al. 2009). Another reason PEG was used was to block active sites where it may interfere with either oxygen transport or other small molecules to hinder oxygenation. One such example was PEGylation through S-nitrosylation at the Cys-β93 to reduce NO scavenging (Asanuma et al. 2007). The most common reaction used to PEGylate Hb is the 2-iminothiolane (IMT) reaction that involves the production of maleimido-PEG and then PEGylated Hb through the reaction with activated thiol groups (Iafelice et al. 2007). Other synthetic polymers used were polyamide and hydrogel-like polymers. Polysaccharides were also conjugated to the Hb such as dextran (Tam et al. 1976; Wong 1988) and heparin (Chauvierre et al. 2007). The conjugation reaction involves the oxidation of the end terminal sugars by inducing ring-opening and subsequent oxidation through sodium periodate to form dialdehyde groups. This technique was used extensively by Eike and Palmer to form a wide range of HbOCs, based on oxidized saccharide conjugates of Hb (Eike 2005; Eike and Palmer 2006).

Clinically, protein-based HbOCs were proven to ameliorate blood loss, and many are in phase III clinical trials or have been stopped at or before phase III clinical trials. No product is as of yet approved for general trauma use in the United States. A large concern is the antigenicity of Hb, especially when sources other than allogenic sources are used including xenogenic, microbial, and even recombinant. The concern for recombinant lies not in the sequence of the Hb, but in the resulting

posttranslational modifications. This is also a concern when polymerized and conjugated with polymers to increase size. For polymers that are not antiopsonizing or having similar moieties with commonly exposed antigenic conformations, the concern is greater. The problems of using Hb still abound despite improvements. The side effects of NO scavenging and oxidative free radical formation were not blocked adequately using the approaches. The problem of high oxygen affinity was mitigated with the incorporation of SOD or other antioxidants such as ascorbic acid, but did not oxygenate tissues in the range comparable to that of RBCs. It is clear that proteins and polymerized or otherwise modified proteins can be created in nanoscale proportions to biomimic a cellular activity and also that improvements in the systems themselves need to be made in order to properly biomimic RBCs.

22.3.3.2 Liposomal Carriers

The next generation of HbOCs was encapsulated in liposomes and is referred to in the lipid encapsulated hemoglobin (LEH) in North America. In brief, this system entails the encapsulation of the protein of interest, hemoglobin, within the liposome. The liposome is like a micelle, but with a phospholipid bilayer much like a cell. Unlike in gene therapy, where cationic liposomes are used, anionic liposomes are commonly employed for artificial cells because of the increased circulation time (Awasthi et al. 2004). A major drawback for anionic lipids is complement activation (Szebeni et al. 1996); however, Chang and Lister (1990) showed that complement activation varied with manufacturers. To mitigate the problem, a new lipid was fabricated by Sou et al. (2003)—1,5-diplamitoly-L-glutamate-N-succinic acid. Supermicron liposome Hb carriers were initially fabricated by Chang et al. (1969) and submicron lipid vesicles were reported by Djordjevich and Miller. The result was an increase in circulation time compared to supermicron dimensions of the same system (Djordjevich and Miller 1980). Many modifications were made to enhance the circulation time but the most success was obtained through PEGylation by Philips et al. (1999) up to 30 h (Phillips et al. 1999).

A key drawback in LEH systems is the high percent conversion into metHb during the fabrication process. In a common pasteuration process demonstrated by Tsuchida et al., Hb was converted to carbonyl Hb (HbCO) to reduce formation of metHb at 60°C for 10 h. The process, however, also removes and inactivates enzymes and antioxidant essential for proper dissociation of oxygen from Hb (Tsuchida 1994). This also results in reduced oxygenation of tissues in the LEH system due to inability of reducing agents to cross the lipid bilayer.

Another problem for LEH is the necessity for submicron particles, nanoparticles, in order to maximize circulation time and reduce reticuloendothelial clearance (Liu et al 1992)—less than 200 nm but greater than 60 nm. The problem lies in lower encapsulation efficiency with smaller nanoparticles. Using the PEGylated LEH as a platform, PEGylated phosphatidylethanolamines (PEG-PE) were used to produced LEH resulting in increased encapsulation efficiency and circulation time (Awasthi et al. 2004). As expected the majority of the LEH were cleared by the reticuloendothelial system.

To administer the LEH, an emulsifier is needed that may be antigenic or act as haptens. In recent research, human serum albumins (HSA) have been coadministered with LEH. Sakai et al. (2004) used recombinant HSA to coadminister with LEH and demonstrated safe and effective for up to 50% hemodilution in rats. The same complications that may occur due to exposure of proteins not form autologous sources may play a role in second infusions.

The LEH system is fraught with problems that have yet to be addressed totally. Each system produced has sought to address one problem at a time and thus no marketable product performing on par with RBCs has been developed. Many of the problems in the system lie with the fabrication process itself. Through the progression of the research, many points of interest was learned including the ideal size range to reduce uptake and clearance by the reticuloendothelial system as well as insight into the complement activation not seen with the Hb, polymeric Hb, and modified Hb systems. The latter may be due to the lack of trials involving reperfusion and testing for immune system reactions toward the Hb-based products.

22.3.3.3 Polymeric Carriers

Stemming from adding polymeric chains directly to either polymerized or single Hb units, the idea of encapsulating Hb in polymer nanoparticles is at the forefront of research today. The main drives toward the polymer use are due to the disadvantages of the liposomal carriers and the wish to avoid complications of free floating Hb. The basic principle is to optimize a high surface area to volume ratio to expose Hb to plasma conditions, reduce immune system complications, control size of the polymeric nanoparticle to maximize circulation time, and promote the stability of the Hb in active form.

Common polymers employed are poly lactic acid (PLA), poly glycolic acid (PGA), poly caprolactone (PCL), and PEG as well copolymers. The advantages in polymers lie in the ability to control the molecular weight conjugated to the target molecule, control over the chemical properties through the variation of monomers and formation of new monomers, and the ability to avoid antigenic moieties. Polyesters are commonly employed in fabrication of nanoparticles because it has been characterized well, is relatively nontoxic, and degrades into products easily degraded by the body. Some toxicity was seen and was attributed to residue monomers and chemicals involved in the polymerization or other technique (Chang 2007). In many instances, polyesters have been used to deliver both genes (Reul et al. 2009) and drugs (Dailey et al. 2005) throughout the body and can also be used to deliver hemoglobin systemically. PLA was employed by Chang to microencapsulate enzymes in micron scale particles (Chang 1976). Expanding on the need for nanometer dimensions, HbV smaller than 200 nm was fabricated with PLA (Chang and Wong 1972) and PEG-PLA (Chang and Yu 2006). To allow for better functionality of the encapsulated Hb, enzyme systems have been co-encapsulated. Chang (2003) demonstrated that the inclusion of methemoglobin reductase allowed to methemoglobin conversion to Hb in PLA nanoparticles. Other antioxidants have been used as well such as ascorbic acid (Dotsch et al. 1998) and glutathione (Mawatari and Murakami 2004) to reduce methemoglobin conversion. For the greater part, the paradigm is to use thiol group containing compounds to reduce methemoglobin conversion.

22.4 Conclusion

Artificial cells and the utilization of nanomaterials has made great strides in the past decade, and the continued refinement of nanotechnology holds a lot of promise for future developments in drug delivery and tissue regeneration. Carbon nanotubes, metal oxides, liposomes, and polymers all have unique properties of which can be taken advantage to engineer functional artificial cells. The small size of nanoparticles offers an opportunity for scientists and engineers to create artificial cells, as well as matrices for tissue regeneration similar to the biological extracellular matrix (Goldberg et al. 2007).

For the RBC substitute system, there are a variety of branches of nanobiomaterials may be applied and developed to advance research. By modifying outer protein vesicles, "self" moieties may be added to reduce immune responses. The need to develop phospholipids to produce liposomes that do not require emulsifiers and is able to have a low methemoglobin conversion rate as well as being innate to the immune system is apparent. As for polymer science, new polymers or copolymers need to be fabricated and tailored to increase the circulation time, avoid immune response, and allow for greater amount of exposed Hb.

Although nanomaterials and their use for the development of artificial cells has uncovered numerous possibilities for solving present medical issues, a uniform system for assessing the risk to the human body that nanomaterials may pose has not been established. The cytotoxicity of nanomaterials in artificial cells must be evaluated in each stage of their life cycle: development, production, use, and disposal (Tervonen et al. 2009). Most studies have shown that artificial cells composed of polymeric nanomaterials do not have adverse affects on the body and are relatively benign (Kabanov 2006). However, because there is so much that is unknown about the long-term physical, chemical, and biological impact of nanomaterials on biological cells and because there is so much variability in the types of materials and techniques used in the engineering of artificial cells, a classification system based on risk-assessment needs to be established.

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