CHAPTER 2

Basic Principles

In this chapter, the basic principles of artificial cells are described and illustrated by a few of the numerous studies and references in the field. Specific areas with detailed examples and references will follow in the later chapters.

2.1. Basic Features of Artificial Cells

The initial research on artificial cells forms the basic principles of artificial cells, principles that have been extended for use in many areas by many groups. Indeed, as stated in the first monograph entitled *Artificial Cells* (Chang, 1972a): *"Artificial cell is not a specific physical entity. It is an idea involving the preparation of artificial structures of cellular dimensions for possible replacement or supplement of deficient cell functions. It is clear that different approaches can be used to demonstrate this idea."*

Basic features of early artificial cells

Earlier artificial cells have some of the simpler properties of biological cells (Fig. 2.1). Some examples of the basic features are:

(1) The membrane of an artificial cell separates its content from the outside. At the same time, the membrane can be prepared such that it can selectively allow different types of molecules to cross it. This ranges from membrane that does not allow any molecules to cross it to those that allow even very large molecules like proteins to cross it. In between these two extremes, there are artificial cell

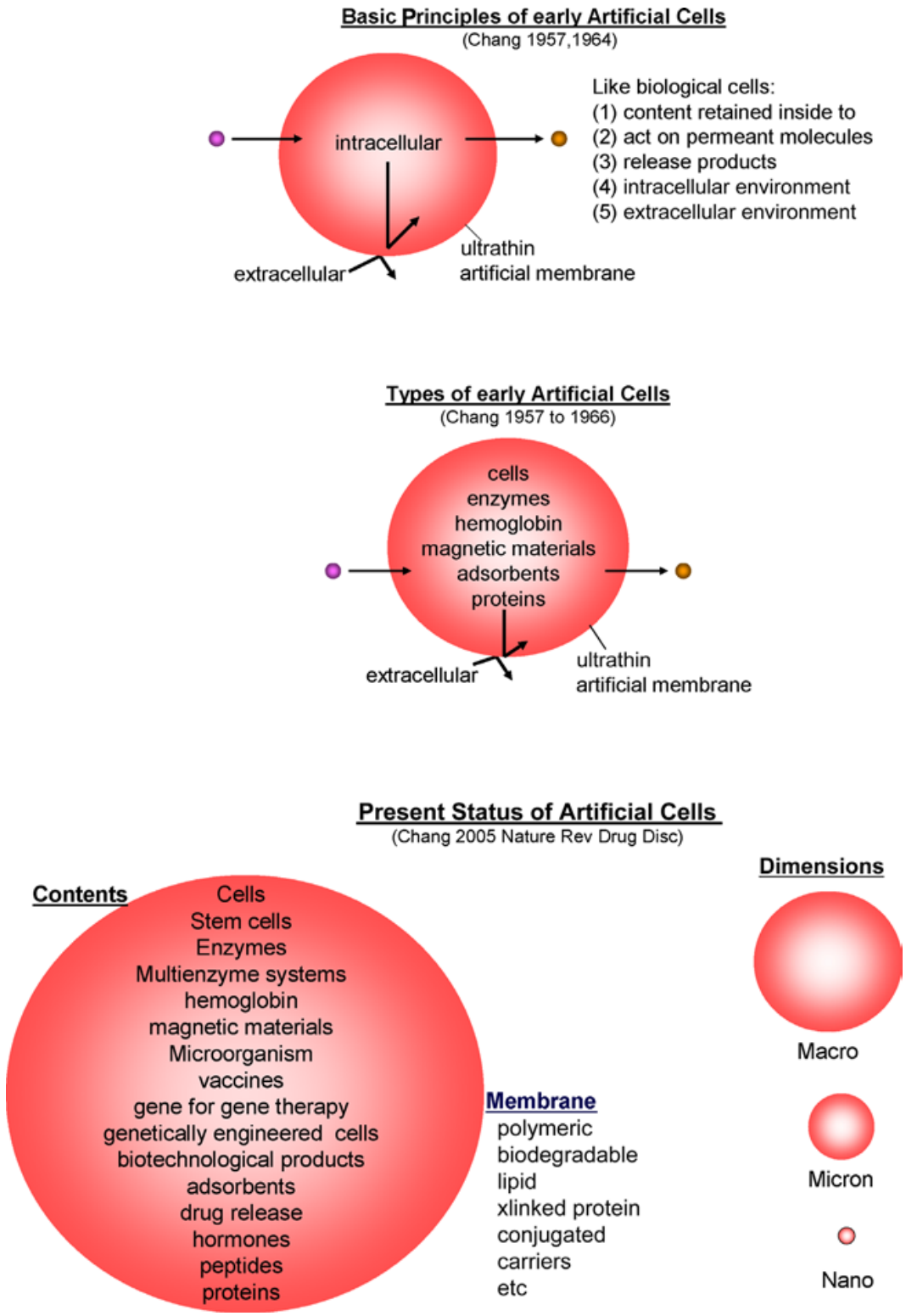

Fig. 2.1. *Upper*: Basic principles of early artificial cells. *Middle*: Different types of early artificial cells based on these basic principles. *Lower*: Present status of artificial cells with wide variations in contents, membrane material and dimensions.

membranes that restrict the movement of molecules according to molecular size, lipid solubility, affinity to carrier mechanisms, etc.

(2) Artificial cell membranes can be very thin, yet strong and have a large surface area. Thus, 10 ml of 20 μm diameter artificial cells has a total surface area of 2500 cm^2. This is the same as the total membrane surface area of an artificial kidney machine. In addition, an artificial cell membrane is 100 times thinner than that of an artificial kidney membrane. This means that smaller molecules can move across 10 ml of 20 μm diameter artificial cells 100 times faster than that across an artificial kidney machine (Chang, 1966). The microscopic size of artificial cells also allows material to diffuse rapidly inside the artificial cells.

(3) Artificial cells can contain the same biological material as biological cells. In addition, they are more versatile since adsorbents, magnetic materials, cells, drugs and other material can also be included separately or in combination (Fig. 2.1).

Present status of the basic features of artificial cells of macro, micron, nano and molecular dimensions

The general principles of artificial cells can form the basis of a large number of artificial systems (Fig. 2.1). In addition to being of cellular dimensions in the micron range, they can also be in the macro range, nano range or molecular range. Furthermore, the membrane material includes polymer, biodegradable polymer, lipid, crosslinked protein, lipid-polymer complex, lipid-protein complex and membrane with transport carriers. Artificial cells can contain an unlimited variety of material individually or in combinations (Fig. 2.1). These include cells, stem cells, enzymes, multienzyme systems, hemoglobin, magnetic materials, microorganisms, vaccines, genes for gene therapy, genetically engineered cells, adsorbents, drugs, hormones, peptides, proteins and others.

The following is a brief overview of some of examples that illustrate the basic principles. Later chapters contain detailed references and descriptions.

2.2. Nanotechnology and Nanobiotechnology

There is much recent interest in nanotechnology. Nanotechnology is a large and complex area that embraces many diverse approaches. One of these is to make the original artificial cells smaller using the same basic principles and method. This includes biodegradable nanoparticles, nanospheres and nanocapsules. Examples include nano artificial red blood cells with lipid membrane (Djordjevich and Miller, 1980) or biodegradable polymeric membranes nano artificial red blood cells (WP Yu and Chang, 1994) (Fig. 1.1). A later section will summarize other examples used in drug delivery systems.

Nanobiotechnology and artificial cells

Nanobiotechnology is the assembling of biological molecules into nanodimension structures, membranes with nanodimension thickness or nanotubules with nanodimension diameter.

The first nanobiotechnology approach reported is the crosslinking of hemoglobin into ultrathin polyhemoglobin (PolyHb) membrane with nanodimension thickness (Chang, 1964, 1965) (Fig. 2.2). This is used to form the membrane of artificial red blood cells (Chang, 1964, 1965). If the emulsion is made very small, then the whole submicron artificial cells can be crosslinked into PolyHb of nanodimension. Glutaraldehyde can crosslink hemoglobin to form soluble nanodimension PolyHb, each consisting of an assembly of 4–5 hemoglobin molecules (Chang, 1971b) (Fig. 2.2).

Two groups have independently developed this 1971 basic method of glutaraldehyde crosslinking for clinical use. One is glutaraldehyde human PolyHb (PolyHb) (Gould *et al.*, 1998; Gould *et al.*, 2002). Their phase III clinical trial shows that this PolyHb can replace blood lost in trauma surgery by keeping the blood hemoglobin at an acceptable level. The second PolyHb is glutaraldehyde-crosslinked bovine PolyHb which has been tested in Phase III clinical trials (Pearce and Gawryl, 1998; Sprung *et al.*, 2002). South Africa has approved this PolyHb for routine clinical use in patients. Unlike red blood cells, there is no blood group, and thus PolyHb can be given on the spot, without

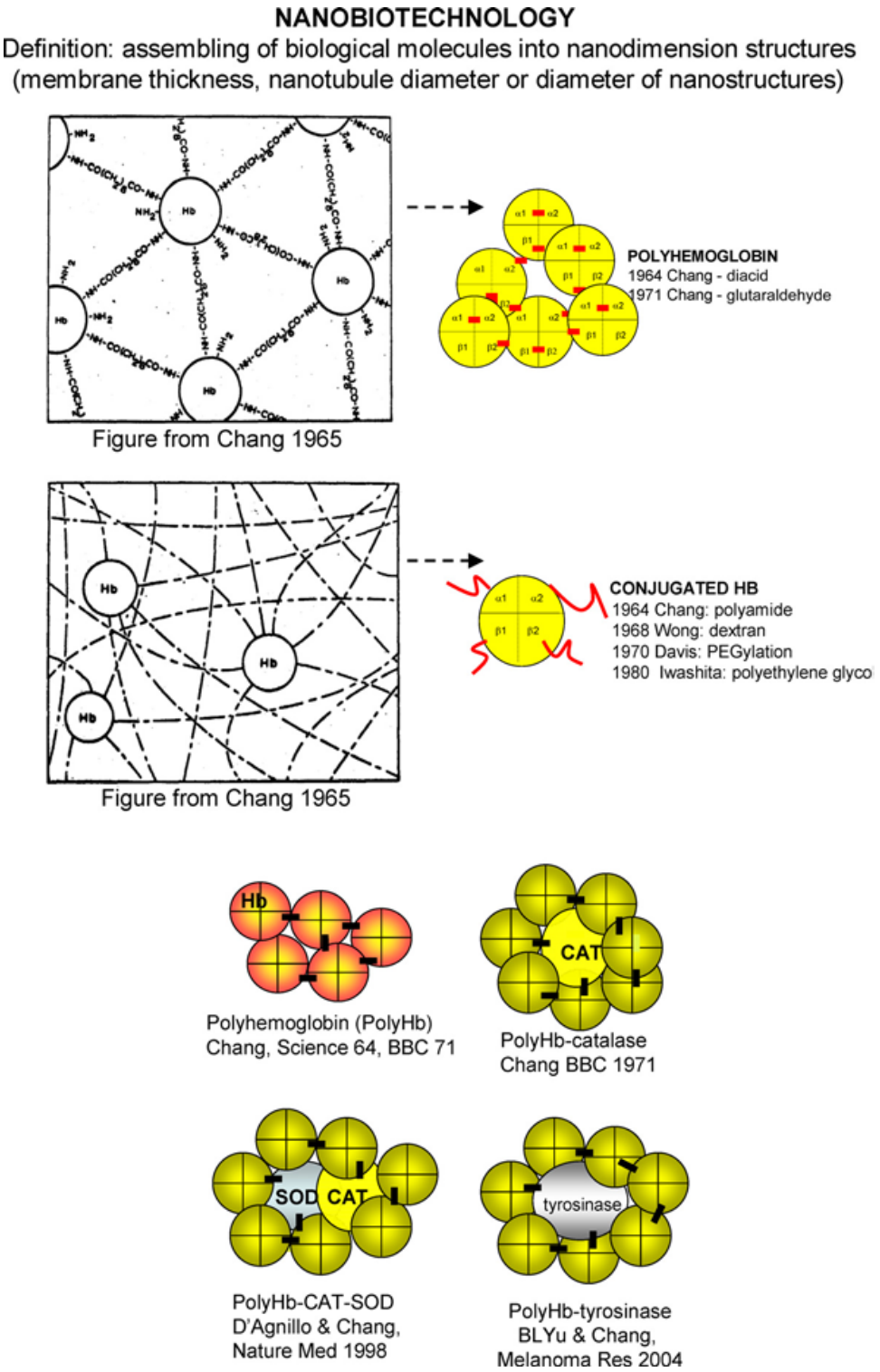

Fig. 2.2. Nano artificial cells can be prepared in nanodimensions as membrane-enclosed nano artificial cells or by the use of nanobiotechnology to assemble biological molecules together into nanodimension structures. *Upper*: An example of assembling of biological molecules to form polyHb and conjugated Hb. *Lower*: Examples of different types of nanobiotechnology-based polyHb-enzymes.

waiting for typing and crossmatching in the hospital. They are also free from infective agents such as HIV, hepatitis C, bacteria, parasites and so on. Furthermore, whereas donor blood has to be stored at 4°C and is only good for 42 days, PolyHb can be stored at room temperature for more than a year. Thus, PolyHb can have important uses in a number of clinical conditions notably for surgery.

Nanobiotechnology and the assembling of hemoglobin with enzymes that remove oxygen radicals

As PolyHbs can be kept at room temperature and used immediately on the spot, they can have potential for use in treating severe bleeding (hemorrhagic shock). However, the process must be carried out fast because if delay occurs, the PolyHb alone might result in the production of oxygen radicals that cause tissue injury (ischemia-reperfusion injuries). Antioxidant enzymes normally present in red blood cells are not enough to prevent this problem. We use glutaraldehyde crosslinking to assemble a nanobiotechnology complex of PolyHb-SOD-CAT by crosslinking hemoglobin, superoxide dismutase and catalase (D'Agnillo and Chang, 1998) (Fig. 2.2). In this way, one can increase the antioxidant enzymes to a much higher level than those in red blood cells.

Obstruction of arteries due to clots or other causes can result in stroke or heart attack (myocardial infarction). Being a solution, PolyHb can more easily perfuse partially obstructed vessels. However, if there is a prolonged lack of oxygen, reperfusion with PolyHb alone may give rise to damaging oxygen radicals, resulting in ischemia-reperfusion injuries. Thus, in a rat stroke model, after 60 min of ischemia, reperfusion with PolyHb resulted in a significant increase in the breakdown of the blood-brain barrier and an increase in brain water (brain edema) (Powanda and Chang, 2002). On the other hand, polyHb-SOD-CAT did not result in these adverse changes (Powanda and Chang, 2002).

Nanobiotechnology for the assembling of hemoglobin with other enzymes

Abnormal microcirculation in tumor leads to a decrease in perfusion by oxygen carrying red blood cells (Pearce and Gawryl, 1998). PolyHb

can more easily perfuse the abnormal microcirculation of tumors to supply oxygen needed for chemotherapy or radiation therapy. With a circulation half-time of 24 h, the effect can be adjusted to the duration of the chemotherapy or radiation therapy. When used together with chemotherapy, PolyHb decreases the growth of tumor and increases the lifespan in a rat model of gliosarcoma brain tumor (Pearce and Gawryl, 1998). We have recently crosslinked tyrosinase with hemoglobin to form a soluble PolyHb-tyrosinase complex (BL Yu and Chang, 2004) (Fig. 2.2). This has the dual function of supplying the needed oxygen and at the same time lowering the systemic levels of tyrosine needed for the growth of melanoma. Intravenous injections delayed the growth of the melanoma without causing adverse effects in the treated animals (BL Yu and Chang, 2004).

Conjugation of polymer with proteins

In the presence of diamine, sebacyl chloride crosslinks hemoglobin with polyamide to form conjugated hemoglobin (Chang, 1964,1965) (Fig. 2.2). This can be in the form of nanothickness artificial cell membranes or conjugated Hb nanospheres. An extension of this is the crosslinking of single enzyme or single hemoglobin molecule to soluble polymers (Tam *et al.*, 1976; Duncan, 2003; Li, Zhang & Liu, 2005; Winslow, 2006) (Fig. 2.2). Promising Phase II clinical trials are ongoing (Winslow, 2006). This extension is not nanobiotechnology since *conjugation of single biological molecule is not the same process as* assembling of biological molecules.

2.3. Cell Homogenate, Organelle, Enzymes and Multienzyme Systems

Cell homogenate

As discussed in Chapter 1, artificial cells were first prepared by replacing the membrane of biological cells with artificial membranes (Chang, 1957). For example, an artificial membrane replaces the red blood cell membrane, but the content is the same as that in the original red blood cells. The same principle can also be applied to other types of biological cells. For example, we have prepared artificial

cells containing hepatocyte microsomes and cytosol (Yuan and Chang, 1986).

Enzymes and enzyme therapy

A large array of enzyme systems is present in the cell homogenates. Simpler artificial cells can be made to contain only one enzyme (Chang, 1964, 1972) (Fig. 2.3). The enclosed enzyme would not leak

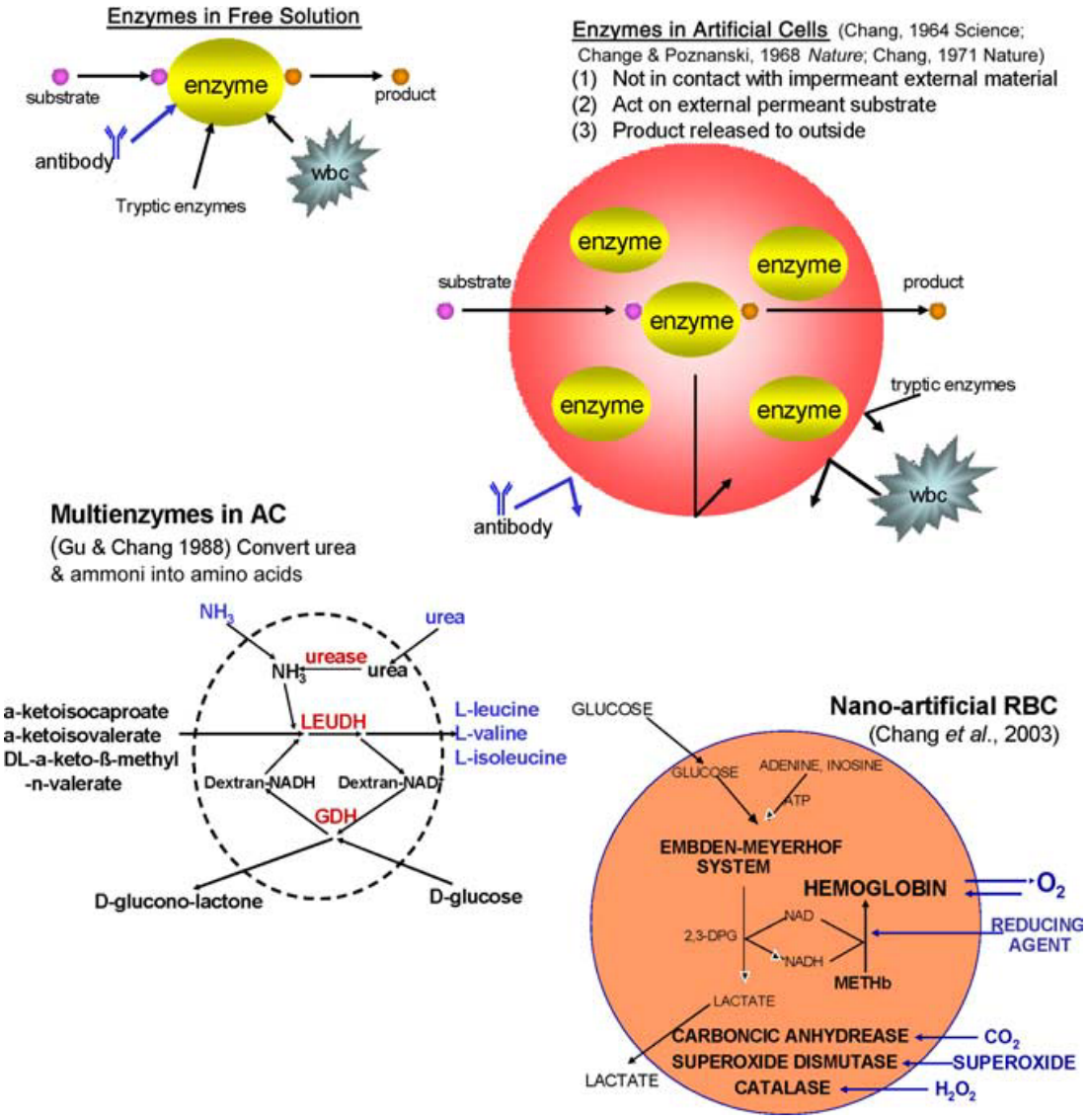

Fig. 2.3. *Upper left*: Problems related to injection of enzymes in free solution. *Upper right*: Enzymes inside artificial cells no longer have these problems. *Lower left*: Artificial cells can be prepared with multistep enzyme systems with recyling of cofactors. *Lower right*: Nano artificial red cells contain all the enzymes of red blood cells.

out, but can act on external permeant substrates. This would avoid protein sensitization, anaphylactic reaction, or antibody production with repeated injections (Fig. 2.3). Implanted urease artificial cells convert systemic urea into ammonia (Chang, 1964, 1965). Implanting artificial cells containing catalase can replace the defective enzyme in mice with a congenital catalase defect — acatalasemia (Chang and Poznanski, 1968). The artificial cells protect the enclosed enzyme from immunological reactions (Poznanski and Chang, 1974). Artificial cells containing asparaginase implanted into mice delay the onset and growth of lymphosarcoma (Chang, 1971).

Giving enzyme artificial cells by mouth obviates the need for repeated injections. For example, artificial cells containing urease and ammonia adsorbent can lower the systemic urea level (Chang, 1972a). In Lesch-Nyhan disease, enzyme defect resulted in the elavation of hypoxanthine to toxic levels. Given by mouth, artificial cells containing xanthine oxidase lowers the toxic systemic hypoxanthine levels in an infant with this disease (Chang, 1989; Palmour *et al.*, 1989). Phenylketonuria is a more common congenital enzyme defect. Artificial cells containing phenylalanine ammonia lyase, given by mouth, lower the systemic phenylalanine levels in phenylketonuria [PKU] in rats (Bourget and Chang, 1986). This leads to investigation into recombinant sources of this enzyme (Sarkissian *et al.*, 1999; Liu *et al.*, 2002).

Multienzyme systems with cofactor recycling

Most enzymes in the body function as multienzyme systems with cofactor recycling. After basic research on artificial cells containing multienzyme systems (Chang, 1985a), we looked into their possible use. Thus, artificial cells containing three different enzymes can convert metabolic wastes like urea and ammonia into essential amino acids (Gu and Chang, 1988) (Fig.2.3). The needed cofactor, NADH, can be recycled and retained inside the artificial cells by crosslinking to dextran or by using a lipid-polymer membrane. All the multienzyme systems in red blood cells can be included inside nanodimension artificial red blood cells (Chang *et al.*, 2003) (Fig. 2.3).

2.4. Artificial Cells Containing Intracelluar Compartments

Biological cells contain intracellular organelles. This allows separate compartments inside the cells to carry out specific functions more effectively. We have prepared artificial cells that also contain intracellular compartments (Chang 1965, 1972a; Chang *et al.*, 1966)

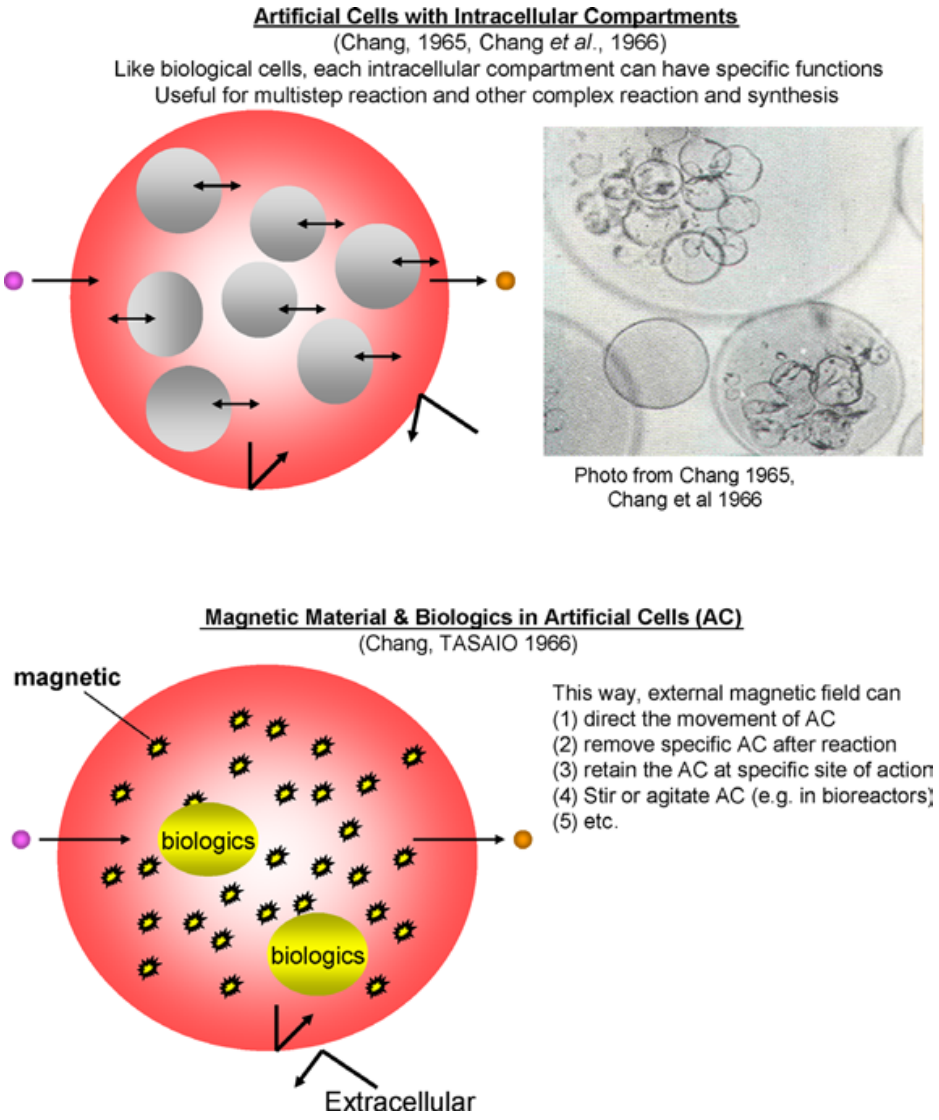

Fig. 2.4. *Upper*: Artificial cells can be prepared with intracellular multi-compartments. *Lower*: Artificial cells containing biologics can also contain magnetic material allowing artificial cells to be site directed. Both principles are being extended by many groups and are being used in different areas of application and research.

(Fig. 2.4). Specific enzyme systems or other biologically active systems can be enclosed separately or in combination in each of these intracellular compartments to allow for more efficient stepwise functions.

2.5. Artificial Cells Containing Biologics and Magnetic Material

When magnetic material is included in artificial cells containing biological materials, one can use an external magnetic field to direct the artificial cells (Chang, 1966) (Fig. 2.4). This principle is now being used very extensively in bioreactors, in removing specific materials from a mixture as in diagnositcs kits, drug delivery systems and other areas of application.

2.6. Cells, Islets, Stem Cells, Genetically-engineered Cells and Microorganisms

Artificial cells containing cells

The first artificial cells containing intact biological cells were reported in 1964 based on a drop method (Chang, 1964), and it was proposed that "*protected from immunological process, encapsulated endocrine cells might survive and maintain an effective supply of hormone*" (1965, Chang *et al.*, 1966) (Fig. 2.5).

Chang asked Conaught Laboratory of insulin fame to develop this for use in islet transplantation for diabetes. Later, Sun and his collaborator from Conaught Laboratory developed Chang's original drop method (Chang, 1964,1965,1972a; Chang *et al.*, 1966), using alginate-polylysine-alginate (APA) for the artificial cell membranes (Lim and Sun, 1980). They showed that after implantation, the insulin secreting islets inside the artificial cells indeed remained viable and continued to secrete insulin to control the glucose levels of diabetic rats (Lim and Sun, 1980).

We have been studying the use of artificial cells containing liver cells (hepatocytes) for liver support. Implanting these increases the

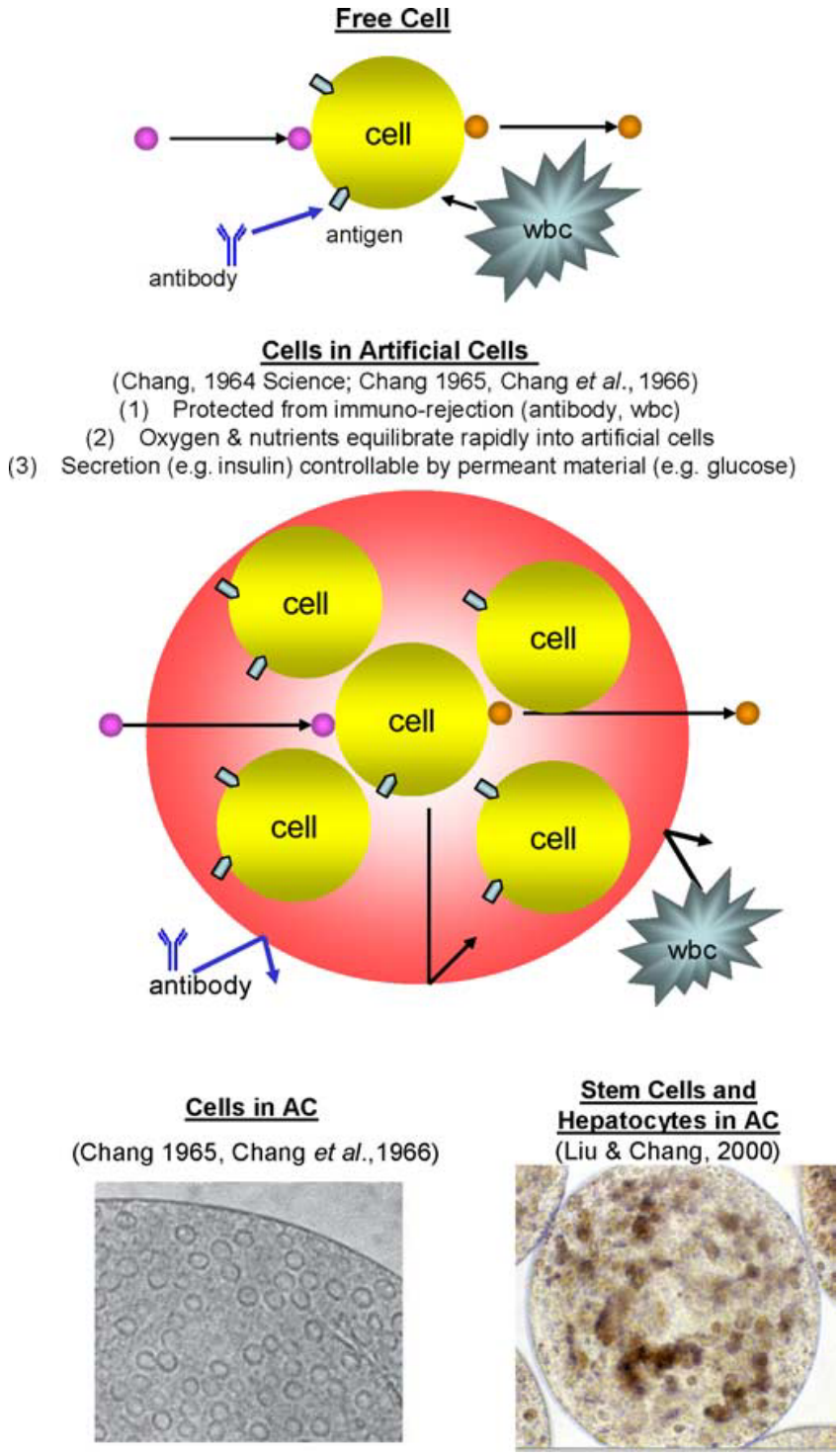

Fig. 2.5. *Upper*: Problems related to injection of free cells. *Middle*: Cells inside larger artificial cells no longer have these problems when injected. *Lower*: Artificial cells containing biological cells. Priniciple has been extended and used by many groups for bioencapsulation of islets, cells, genetically-engineered cells and stem cells (Chang, *Nature Rev. Drug Discovery*, 2005).

survival of acute liver failure rats (Wong and Chang 1986); lowers the high bilirubin level in congenital Gunn rats (Bruni and Chang, 1989); and prevents xenograft rejection (Wong and Chang, 1988). We developed a two-step cell encapsulation method to improve the APA method, resulting in improved survival of implanted cells (Wong and Chang, 1991a). Cell bioencapsulation for cell therapy has been extensively developed by many other groups especially using artificial cells containing endocrine tissues, hepatocytes, genetically-engineered cells and stem cells (Orive *et al.*, 2002; Chang, 2005). This is a very broad area that will be described in much more detail in the later chapters. Below is a brief introduction to the use of this principle for stem cells and genetically-engineered cells.

Stem cells

We used the two-step method and the coencapsulation of stem cells and hepatocytes into artificial cells (Liu and Chang, 2000) (Fig. 2.5). This results in further increase in the viability of encapsulated hepatocytes both in culture and after implantation (Liu and Chang, 2002). An implantation of artificial cells containing both hepatocytes-stem cells into Gunn rats lowers the systemic bilirubin levels, the low level being maintained for two months (Liu and Chang, 2003). Without stem cells, the implanted hepatocytes in artificial cells can only be maintained at the low level for one month. What is even more exciting is our recent finding using artificial cells containing only bone marrow stem cells and no hepatocytes. The control group of rats with 90% of their liver surgically removed did not survive. Unlike free stem cells, an intraperitoneal injection of artificial cells containing bone marrow stem cells alone results in long-term survival (Liu and Chang, 2006). Along with this, the livers regenerate and return to their normal weights.

Genetically-engineered cells

Many groups have carried out extensive research on artificial cells containing genetically-engineered cells. This more recent and very important area will be discussed in detail in a later chapter, including

potential applications in amyotrophic lateral sclerosis, dwarfism, pain treatment, IgG_1 plasmacytosis, hemophilia B, Parkinsonism and axotomized septal cholinergic neurons, tumor suppression and other areas (Basic *et al.*, 1996; Tan *et al.*, 1996; Al-Hendy *et al.*, 1996; Okada *et al.*, 1997; Dalle *et al.*, 1999; Saitoh *et al.*, 1995; Hagihara *et al.*, 1997; Winn *et al.*, 1994; Aebischer *et al.*, 1996; Bloch *et al.*, 2004; Bachoud-Levi *et al.*, 2000; Xu *et al.*, 2002; Cirone *et al.*, 2002). To avoid the need for implantation, we studied the oral use of artificial cells containing genetically-engineered nonpathogenic *E. coli* DH5 cells to lower systemic urea in renal failure rats (Prakash and Chang, 1996; Chang, 1997).

2.7. Artificial Cells Containing Bioadsorbents

As mentioned earlier, the microscopic dimensions of artificial cells result in a large surface-to-volume relationship. This, together with the ultrathin membranes, allow artificial cells containing bioadsorbents to be much more effective when compared to standard hemodialysis in removing toxins and drugs from the blood of patients (Chang, 1966, 1969a, 1975g).

The most common routine application of this approach is the use of microscopic polymeric artificial cells encapsulating activated charcoal (Chang, 1969, 1973a,b, 1975g) (Fig. 2.6). Its use solves the major problems of release of embolizing particles and damage to blood cells when bioadsorbents are used without the artificial cell membranes (Fig. 8). The first successful application was in suicidal overdose patients (Chang *et al.*, 1973a,b). Since then, this has become a routine treatment worldwide for acute poisoning in adults and children, especially in cases of suicidal overdose (Chang, 1975b, 1975c; Winchester, 1988; Singh *et al.*, 2004; Lin *et al.*, 2004; Peng *et al.*, 2004; Lopez *et al.*, 2002; Kawasahi *et al.*, 2000; Lin *et al.*, 2002; Tominaga, 1997). The treatment is particularly useful in places where dialysis machines are not readily available.

The approach is also effective in removing toxic products in kidney failure patients (uremia), resulting in the relief of uremic symptoms (Chang *et al.*, 1971a; Chang, 1975g; Winchester, 1988). Components

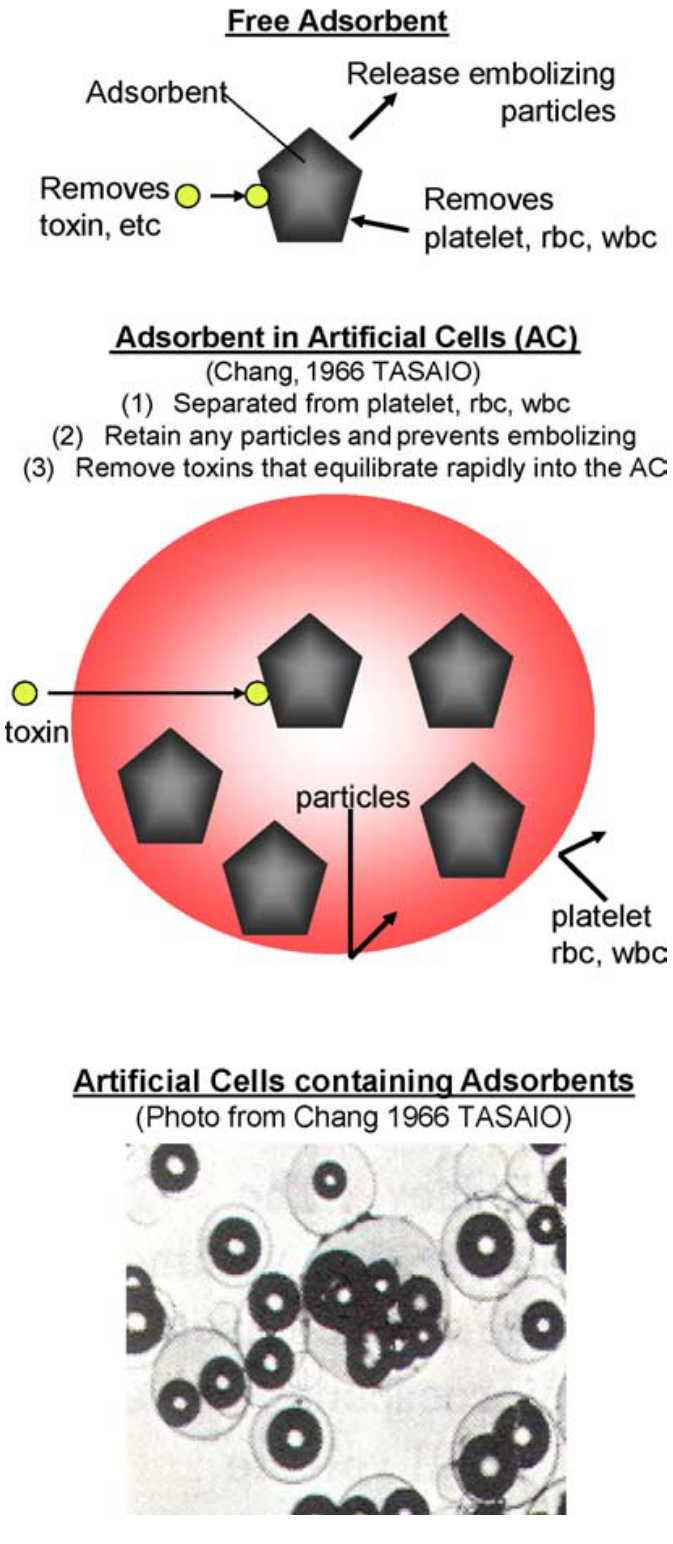

Fig. 2.6. *Upper*: Problems related to adsorbents when used to remove toxin from blood. *Middle*: Adsorbents inside artificial cells no longer have these problems and therefore can be used in hemoperfusion to remove toxins from blood. *Lower*: Adsorbent inside artificial cells. Since its first clinical use as artificial cells containing activated charcoal in hemoperfusion in patients, it is now a routine method for the treatment of acute poisoning.

for the removal of other uremic wastes need to be developed. The approach has also proved to be effective in removing toxic molecules in patients with severe liver failure, resulting in the recovery of consciousness of grade 4 hepatic coma patients (Chang, 1972b, 1975g; Gazzard *et al.*, 1974). Detoxification is only one of the functions of the liver, and this approach is being used as the detoxification component of hybrid liver support systems that are being developed (Liu *et al.*, 2001).

The success in the clinical uses of artificial cells containing bioadsorbents for detoxification has led to an increasing interest in research and development in many other areas. One of these is in artificial cells containing immuoadsorbents (Chang, 1980d).

2.8. Research on Membrane Model Systems

Since the membrane compositions of artificial cells can be varied at will, they can serve as membrane model systems. Indeed, work in this laboratory shows that it is possible to vary the membrane properties as to porosity, thickness, charge, lipid content, protein content, mucopolysaccharide content, and polymer composition (Chang, 1964, 1965, 1972a). We have used artificial cells to study the biophysics of membrane transport (Chang and Poznansky, 1968c); the relationship of surface properties to survival in circulation (Chang, 1965, 1972a; Chang *et al.*, 1967b); and the relationship of physicochemical properties to the effect on coagulation and formed elements of blood (Chang *et al.*, 1967b; Chang, 1969a). Other workers have made use of these artificial cells in the study of the mechanical and electrical properties of membranes (Jay and Edwards, 1968; Jay and Burton, 1969; Jay and Sivertz, 1969).

Bangham and his co-workers prepared liposomes that are liquid crystal microspheres, each consisting of concentric shells of bimolecular lipid layers as a model system (Bangham *et al.*, 1965). Mueller and Rudin (1968) adopted the Chang procedure for the preparation of artificial cells (Chang, 1964) to prepare artificial cells having only a single bilayer lipid membrane. Since biological cell membranes consist of both protein and lipids, we

have prepared artificial cells with membrane of lipid-polymer or lipid-protein for membrane transport studies (Chang, 1969d). This also includes the incorporation of macrocyclic molecules into the membrane as a carrier transport mechanism (Chang, 1969d). Another approach is to incorporate Na^+-K^+-ATPase into the lipid-polymer artificial cell membrane (Rosenthal & Chang, 1980). Since then, there have been very extensive studies on the inclusion of many different types of transport system or targeting agents or other materials into different types of artificial cell membranes (Torchilin, 2005; Chang, 2005).

2.9. Cell Physiology

In cell physiology, there is much research on the origin of cellular responses. One important area is the location of the triggering mechanisms and whether they are located at the cell surface, in intracellular organelles, or in the soluble constituents of the cytoplasm. It has been suggested (Chang, 1972a) that the enclosure of cell contents in artificial cells might be one way of studying this. It would be interesting to test the sites of action of certain hormones; changes in extracellular electrolytes; and substances causing the release of certain intracellular material. This would throw light on whether the substance acts on specific surface receptors, or only indirectly affects the intracellular material, or whether it directly affects the intracellular material.

2.10. Drug Delivery

Polymeric semipermeable microcapsules

Luzzi (1970a) used nylon membrane artificial cells as reported earlier (Chang, 1964) to microencapsulate drugs for slow release. Others have also extended this approach. However, the modern approaches in drug delivery systems are based on nanotechnology. This is to prepare much smaller artificial cells in nanodimensions.

Biodegradable polymeric artificial cells, nanoparticles, nanocapsules

Biodegradable membrane artificial cells have been prepared to contain enzymes, hormones, vaccines, and other biologicals (Chang, 1976a). The polylactide polymer can degrade in the body into lactic acid and finally into water and carbon dioxide. In the same study, variations in preparation can result in artificial cells that release insulin at different rates (Chang, 1976a). Biodegradable drug delivery systems are now used widely in different forms, ranging from microscopic to nanodimensions (LaVan *et al.*, 2002). They are also known as nanoparticles, nanocapsules, polymersomes, nanotubules, etc.

Liposomes — lipid membrane artificial cells

Bangham first reported the preparation of liposome consisting of microspheres of hundreds of concentric lipid bilayers — multi-lamellar (Bangham *et al.*, 1965). They used these as a membrane model for basic membrane research. When Gregoriadis completed his Ph.D. in biochemistry at McGill, he came to see me to discuss his future areas of research. I encouraged him to look into different ways of forming artificial cells for delivery of biologics. His subsequent research in England on the use of liposomes as drug delivery systems opened a whole new approach (Gregoriadis, 1976).

The large amount of lipid in the original multilamellar liposomes (Bangham *et al.*, 1965) limits the amount of drugs or biologics that can be enclosed. Thus, the basic principles and methods of artificial cells (Chang, 1957, 1964) were extended by researchers into an "ether evaporation method" to form single bilayer (unilamellar) lipid membrane liposomes (Deamer and Bangham, 1976). Much research and development since then have resulted in liposomes being used extensively as pharmaceutical drug carriers (Torchilin, 2005). The modern single bilayer lipid vesicles are actually single bilayer lipid membrane artificial cells. However, even now, there is still reluctance in using the term "artificial cells" and the term "liposome" continues to

be used. However, some researchers and research groups are starting to call these "artificial cells."

2.11. Other Systems

As suggested earlier (Chang 1974, 1965, 1972a), the general principles of artificial cells could be explored as follows. Artificial cells containing radioactive isotopes or antimetabolites might be used for intraarterial injection into tumors. In this case, some of the microcapsules might lodge at the tumor site, while others would be carried by lymphatic channels to act on tumor cells that have metastasized to the regional lymph nodes. Artificial cells containing radiopaque material would provide a contrast medium. Provided they can circulate readily in the bloodstream, they might be used as vehicles for contrast materials in angiography. Artificial cells containing highly magnetic alloys might provide a useful preparation for the measurement of blood flow in unopened vessels by electromagnetic techniques. If membranes of crosslinked protein can be made to retain the immunological characteristics of the protein, there might be a place for these in serological studies or target drug delivery.