CHAPTER 11

Perspectives on the Future of Artificial Cells as Suggested by Past Research

11.1. Introduction

In the last 50 years, there have been numerous extensions and developments of the idea of artificial cells around the world. However, as shown by the examples in the last 10 chapters, we have barely touched the surface of the full potential of this idea. Many other extensions and variations in the membrane material, the configurations and the contents are possible. The current international interest in the transfer of technology for industrial development will help accelerate these developments. To do this, there is a need for patents that are based on novel ideas. A characteristic of such developments is that a new development or extension of “artificial cells” tends to be hidden under numerous new names. Some of these are nanoparticle, nanotubule, lipid vesicle, liposome, polymer tethered lipid, polymersome, microcapsule, bioencapsulation, nanocapule, nanosensor, macroenapsulation, polyhemoglobin, conjugated hemoglobin, etc. The result is a fragmentation of the field of artificial cells into different subdivisions, subdisciplines as well as scientific societies that do not interact with one another. Now is the time for researchers from the different areas in this interdisciplinary field to come together to move the field forward. This monograph is an attempt to bring some of these areas together. As the whole field is now so broad and extensive, that it is not possible to cover everything in one monograph. This final chapter can only touch on some of the other extensions and future developments of the idea of artificial cells that
have not been discussed in the previous chapters. There will be more books published on the subject under this book series.

11.2. Membrane Material

At one time, biological cell membrane was thought to be a simple bilayer lipid membrane. However, extensive research by many investigators is beginning to show that it is much more complicated. Indeed, there are indications that protein components play an important structural and functional role (Fig. 11.1). Biological cell membrane is complex and there is still much basic research to be done. Artificial cell membrane is much more simple minded. However, since it can be formed using different types of synthetic or biological material, there are great potential for variations and extensions. The following are some examples (Fig. 11.1): 1) polymer and crosslinked protein membranes; 2) lipid membranes; 3) lipid-polymer complex membranes; 4) albumin, antitgen, antibody or protein complexed membranes; 5) lipid-polymer complexed membrane with cyclic carrier; 6) lipid-polymer complexed membrane with Na-K ATPase and other transport carriers; 7) lipid membrane with transport carriers; and 8) others.

11.3. Polymeric Membrane

*Porosity of polymeric membrane of artificial cells*

The first artificial cells prepared have ultrathin membranes of cellulose nitrate, polyamide, silastic, polystyrene or crosslinked protein (Chang, 1957 to 1972). A silastic membrane is only permeable to gases like oxygen and carbon dioxide and lipophilic molecules. Those with cellulose nitrate or polyamide membranes are permeable to hydrophilic molecules. For instance, experimental analysis shows that a polyamide membrane has an equivalent pore radius of 18–19 Å (Chang, 1965,1972a) (Fig. 11.2). This means that polyamide membranes and cellulose nitrate membranes would exclude protein molecules but allow polypeptides and other smaller molecules to pass through rapidly.
Fig. 11.1. Schematic representation of biological membrane (top). Examples of artificial cell membranes: polymer and cross-linked protein membrane; lipid membranes; lipid-polymer membrane; membrane with albumin, antigen, antibody or other proteins; lipid-polymer membrane with cyclic carrier; lipid-polymer membrane with Na-K-ATPase transport carriers; lipid membrane with transport carriers.
Fig. 11.2. Equivalent pore radius of polyamide membrane artificial cells obtained experimentally, as shown in above graph, is 18–19 Å.

Polymeric membranes with unlimited variations in porosity and molecular weight cut off are available (Fig. 11.3). At the one, polymeric membranes like silastic or polyethylene membrane are not porous and only allow gases and lipophilic molecules to pass through. On the other, macroporous polymeric membranes allow very large protein molecules to pass through. In between the two extremes, different types of polymeric artificial cell membranes can be prepared with different porosity and molecular weight cut off. This would allow for variations in porosity and permeability characteristics to suit different conditions. In addition, we can go beyond just porosity. For instance, one can even fulfill the requirements for those applications that require the removal of large lipophilic molecules but not the smaller hydrophilic molecules or moisture. For instance, adsorbent artificial cells are normally formed by the direct coating of porous ultrathin membranes onto active charcoal microspheres to remove hydrophilic molecules (Chang, 1969a). However, coating with a polyethylene membrane results in
### Molecular exclusion of membranes (Da)
- **Plasmapheresis membrane** (>200,000 Da)
- **Agarose** (> 150,000 Da)
- **Alginate-Polylysine-Alginate** (60,000-70,000 Da)
- **Dialysis membrane** (60,000 Da)
- **Cellulose nitrate or Polyamide** (30,000 Da)
- **Lipid complexed polymer** (100-200 Da)
- **Lipid vesicles or Silastic** (lipophilic)

### Molecular dimensions (Da)
- IgM (950,000 Da)
- Urease (482,700 Da)
- C19 (410,000 Da)
- Fibrinogen (339,000 Da)
- Phenylalanine ammonia lyase (320,000 Da)
- Catalase (247,000 Da)
- C4 (210,000 Da)
- C5 (195,000 Da)
- IgE (190,000 Da)
- Human leucocytes antigens (180,000–210,000 Da)
- C3 (185,000 Da)
- IgA (170,000–720,000 Da)
- C2 (170,000 Da)
- C8 (163,000 Da)
- IgD (160,000 Da)
- IgG (150,000 Da)
- Tyrosinase (128,000 Da)
- C6 (110,000 Da)
- C7 (100,000 Da)
- Transferrin (81,000 Da)
- C9 (79,000 Da)
- Albumin (66,248 Da)
- Hemoglobin (64,000 Da)
- Factor X (55,000 Da)
- TNF (51,000 Da)
- Superoxide dismutase (31,187 Da)
- IL-1β (17,000 Da)
- NGF (13,000 Da)
- C3a (9000 Da)
- Insulin (5733 Da)
- C6a (4000 Da)
- β-Endorphin (3438 Da)
- Glucose (180 Da)
- Tyrosine (163 Da)
- Phenylalanine (147 Da)
- Glutamine (128 Da)
- Asparagine (114 Da)
- Creatinine (113 Da)
- Urea (50 Da)
- Carbon dioxide (44 Da)
- Ammonia (17 Da)
- Oxygen (16 Da)

**Fig. 11.3.** Possible variations in porosity of the polymeric membranes of artificial cells.

Adsorbent artificial cells that can exclude small hydrophilic molecules (e.g. creatinine) and rapidly remove larger lipophilic molecules (e.g. vitamin B) (Sipehia, Bannard and Chang, 1986).

The methods of preparing polymeric membrane artificial cell include: solvent evaporation with emulsion or drop (Chang, 1957, 1964); interfacial polymerization with emulsion or drop (Chang,
1964, 1965, 1972a); solvent evaporation with double emulsion (Chang, 1965, 1972a, 1976a); ultrathin polymeric membrane coating (Chang, 1969a); alginate-polylysine-alginate drop method (Lim and Sun, 1980); self-directed assemblies of copolymer (Discher et al., 1999); and many other extensions and improvements (Calafiore et al., 1999; DeVos et al., 2002; Duvivier-Kali et al., 2001; Sakai et al., 2001; Cruise et al., 1999; Hunkeler et al., 2001; Schuldt and Hunkeler, 2000; Uludag et al., 2000; Dionne et al., 1996; Orive et al., 2003; and many others).

11.4. Lipid Membrane Artificial Cells

Bangham et al. (1965) were the first to prepare liposomes that are liquid crystal microspheres, each consisting of concentric shells of bimolecular lipid layers (Fig. 11.3). This has been an useful membrane model for membrane research and later for use in drug delivery.

Mueller and Rudin (1968) reported that they can use a modification of the standard procedure (Chang, 1964) to prepare artificial cells having only 60–100 Å thickness single bilayer lipid membrane (Fig. 11.4). These are of about 90 μ in diameter and contain red blood cell hemolysate. The bilayer lipid membranes are not strong enough to support these 90 μ-diameter artificial cells and they stay intact.

![Fig. 11.4. Left: Liposomes that are liquid crystal microspheres with concentric shells of bimolecular lipid layers (Bangham et al., 1965). Right: bilayer lipid membrane artificial cells containing hemoglobin of Mueller and Rudin (1968) who wrote that they used a modification of the method of Chang (1964) to prepare this.](image-url)
for only a short time. As described below, one solution is to tether
the lipid membrane onto the ultrathin polymeric membrane of the
artificial cell to form lipid-polymer membrane artificial cells (Chang,
1969d, 1972a). Other workers (Deamer and Bangham, 1976) used a
modification of the “ether evaporation method” (Chang 1957, 1964) to
form smaller single bilayer (unilamellar) lipid membrane artificial cells.
They call these lipid membrane artificial cells, unilamellar liposomes
or lipid vesicles. As will be discussed later, this forms the basis of the
types of lipid vesicle being currently used in drug delivery systems
(Torcillin, 2005).

11.5. Artificial Cells with Lipid-Polymer Membrane and
Incorporation of Macrocyclic Carrier,
NA-K-ATPase and Other Carriers

The exact molecular organization of biological cell membranes is
not known. However, it is known that biological membranes are not
composed of lipids alone and there is increasing evidence that the
protein components play an important structural and functional role.
Korn (1968) even suggested that in the biosynthesis of biological cell
membranes the protein unit of the membrane is formed first, followed
by the addition of the lipid components.

There are three types of lipid-polymer membrane artificial cells
first reported (Fig. 11.5): 1) lipid-polymer membrane with ultrathin
lipid component tethered to the polymeric membrane of artificial
cells (Chang, 1969d, 1972a); 2) addition of macrocyclic carrier
(valinomycin) to the lipid-polymer artificial cell membrane (Rosenthal
and Chang, 1971, 1980); 3) addition of transport carrier, Na-K-ATPase,
to the polymer artificial cell membrane (Rosenthal and Chang, 1980).
A number of ongoing studies by different groups will also be discussed
below.

**Artificial cells with lipid-polymer membrane**

As described in detail in Appendix II, we complex an equimolar
lecithin-cholesterol mixture to the artificial cells membrane (polymeric
or crosslinked protein) (Fig. 11.5) (Chang, 1969d, 1972a). For artificial cells without lipid on the membrane, sodium influx is very high across the porous polymeric membrane, $6 \times 10^{-6}$ mM/h-mm$^2$ (Fig. 11.6). Complexing the polymeric membrane with an
Fig. 11.6. Equilibration of Na$^+$ across large polymeric membrane artificial cells as compared to that across large lipid-polymer membrane artificial cells (Chang, 1969d, 1972a).

An equimolar lecithin-cholesterol mixture reduces the sodium influx to $4 \times 10^{-6}$ mM/h-mm$^2$ (Fig. 11.6). Surprisingly, this sodium flux across the lipid-polymer membrane artificial cells is of the same order of magnitude as that observed by Stein for most natural membranes (Stein, 1967). On the other hand, a study by another group (Pagano and Thompson 1968) showed that across bilayer lipid membranes, the sodium influx is of orders of magnitude smaller than that of biological cell membranes. Thus, lipid-polymer membranes function more like biological membranes as compared to lipid bilayers alone.

**Incorporation of channels into lipid-polymer membrane of artificial cells**

However, lipid-polymer membrane artificial cells by themselves do not show any selectivity between sodium and rubidium (Fig. 11.7a, 11.7b). We incorporated valinomycin in the lipid-polymer membrane by exposing them to valinomycin at a concentration of $5 \times 10^{-6}$ M.
This results in selectivity so that the rubidium influx is enhanced but the sodium influx remains unchanged (Fig. 11.7a, 11.7b) (Rosenthal and Chang, 1971, 1980). Thus, the permeability coefficients of lipid-polymer membrane artificial cells to rubidium is $1.45 \pm 0.56 \times 10^{-6}$ cm/sec. Valinomycin at a concentration of $5 \times 10^{-6}$ M significantly increases this to $4.91 \pm 1.6 \times 10^{-6}$ cm/sec. However, valinomycin has no effect on the sodium influx and it remains unchanged at $3.8 \pm 0.3 \times 10^{-6}$ cm/sec.

This simple study only shows the potential of the incorporation of channel forming material in the membrane of artificial cells. However, this is just the beginning and much can be learned from the ongoing research of molecular biologists. For instance, ion conducting channels in cell membranes are complex protein structures, but
Bayley (1999) showed that only a bundle of α-helices or nanotubular β-strands is needed to carry out the function. They can retain their function even when most of the protein portion is removed from the complex protein structure. This means that researchers can design synthetic channels or redesign natural channels by changing the amino acid sequence and helix orientation. One of the simplest form is a neutral peptide, Ac-(Leu-Ser-Ser-Leu-Leu-Ser-Leu)_3CONH₂, that can function as a voltage-gated channel. Cyclic peptides can form hollow cylindrical nanotubules with possible variations in the lumen of the channel to allow for transport of different types of solutes based on size (Hartgerink J. D. et al., 1998). In the case of lipid membrane artificial cells, lipid vesicles, exposure to α-hemolysin or its
genetically engineered mutants can increase the permeability to large single stranded nucleic acid (Bayley, 1997). This is an example of how progress in molecular biology can be applied to artificial cells. The large amount of work being carried out by molecular biologists can be tapped for formation on different types of channels in artificial cell membranes.

**Incorporation of Na-K-ATPase in the membrane of artificial cells**

The feasibility of incorporating Na-K-ATPase in the membrane of artificial cells was studied (Rosenthal and Chang, 1980). The details of the method can be found in Appendix II. Briefly, large polyamide membrane artificial cells are first formed. Then Na-K-ATPase is extracted from red blood cell membranes and covalently crosslinked to the artificial cell membranes. The ouabain-sensitive ATPase activity per sq. cm of the surface area of the artificial cells is $90–250 \times 10^{-5} \mu\text{moles Pi/h/cm}^2$ (Table 11.1). This is 1–2 orders of magnitude greater than the ouabain-sensitive ATPase activity of human red blood cell membranes of $6 \times 10^{-5} \mu\text{moles Pi/h/cm}^2$ (obtained by Dunham and Glynn, 1961).

Thus, it is possible to incorporate in artificial cell membranes large amounts of Na-K-ATPase normally found associated with biological membranes. Furthermore, the incorporated enzyme retains its enzyme activity. However, this is only a feasibility study and much remains to be done regarding the orientation, transport characteristics, and association with lipids and other membrane components. The need for ATP recycling in artificial cell is now possible (Campbell and Chang, 1975) and this will be discussed in a later section in this chapter. Again,

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Ouabain-sensitive ATPase activity per cm$^2$ of surface area</th>
</tr>
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<tbody>
<tr>
<td>Red blood cells</td>
<td>$6 \times 10^{-5} \mu\text{moles Pi/h/cm}^2$</td>
</tr>
<tr>
<td>Artificial cells with Na-K-ATPase</td>
<td>$90–250 \times 10^{-5} \mu\text{moles Pi/h/cm}^2$</td>
</tr>
</tbody>
</table>
this area will benefit greatly from the ongoing research and progress in the molecular biology of the structure and function of different transport mechanisms. A few examples are highlighted here.

Melkikh and Seleznev (2005) used nonequilibrium statistical models of the active transport of ions in biomembranes. They used this to study models of active transport of ions in biomembranes of different cells, including neurons, muscular cells, bacteria, plants and mitochondria. They found the values of the membrane potential and ion concentrations to be in qualitative agreement with the experimental data. They discussed the requirements for models of active transport of ions in biomembranes (Melkikh and Seleznev 2006). The factors include resting potential, intracellular concentrations of ions, ATP-ADP chemical potentials, reversibility of the ionic pump, correlation between theoretical and experimental data, pump efficiency and effects of blocking the active transport. Barwe et al. (2007) looked at the Janus model of the NA-K-ATPase beta-subunit. Na-K-ATPase is an oligomer consisting of alpha and beta-subunits. The Na-K-ATPase beta-subunit (Na-K-beta) takes part in both the regulation of ion transport activity, and in cell-cell adhesion. These results provide a structural basis for understanding how Na-K-beta links ion transport and cell-cell adhesion. Pitard et al. (1996) studied ATP synthesis using the F_{0}F_{1} ATP synthase from thermophilic Bacillus PS3 reconstituted into liposomes with bacteriorhodopsin. There is also increasing interest in using the principle of the lipid-polymer membrane artificial cells (Chang, 1969a, 1972; Rosenthal and Chang, 1971, 1980) as a model of biological membrane for studying the structure and function of membrane protein and receptors. Thus, Wagner and Tamm (2000) developed a polymer supported lipid bilayer system to reconstitute integral membrane proteins in a laterally mobile form. When reconstituted in bilayers tethered on quartz, the proteins are not mobile. However, on the lipid-polymer system, they found that the two proteins studied can become laterally mobile. They proposed that the lipid-polymer model allows for the interaction of these proteins with the underlying polymer.
Nanobiosensors

Nanobiosensor is an area that is of increasing interest and different approaches are being investigated (Scheller et al., 2001). One of the many approaches is to use biosensors where the lipid bilayer is “tethered” on ultrathin polymeric support to form a lipid-polymer complex. In this form, different “channels” can be inserted into the membrane to allow for selective movement of specific solute for detection, somewhat similar to the principle reported earlier (Chang, 1969d, 1972a; Rosenthal and Chang, 1971, 1980). Another approach that is also possible is to encapsulate enzymes inside artificial cells of microscopic or nanodimension. This way, the product of enzymatic reaction can be followed by florescence or other methods. The ability to prepare artificial cells with intracellular compartmentation (Chang, 1965, 1972a; Chang et al., 1966) would allow multistep enzyme reactions to occur and be detected separately. Depending on the type of reactions being followed, one can use either polymeric membrane artificial cells, lipid membrane artificial cells or lipid-polymer membrane artificial cells.

11.6. Surface Properties of Artificial Cell Membranes

As discussed under the section on blood substitutes, the surface property of artificial cell membranes is important in terms of their biocompatibility and retention in the circulation, as it is in terms of blood compatibility in hemoperfusion systems. Artificial cells for delivery systems also need to have different types of surface properties. Cellulose nitrate membrane artificial cells (Chang, 1957, 1964) contain negative charges due to the carboxyl groups in the polymer. Polyamide nylon membrane artificial cells (Chang et al., 1963, 1966; Chang, 1964) contain both carboxyl and amino groups. With the proper selection of polymer materials, membranes with the desired fixed charges can be obtained. For example, artificial cell membranes with strong negative charge groups have been prepared by using different amounts of sulfonated diamine such as 4,4’-diamine-2,2’-diphenyldisulfonic acid when preparing polyamide
membrane artificial cells (Chang, 1964, 1965, 1972a). The principle of incorporation of surface charges has also been used the preparation of lipid membrane artificial cells, lipid vesicles, drug delivery systems (Gregoriadis, 1976; Torchilin, 2005).

Albumin can bind tightly to the ultrathin collodion membrane of adsorbent artificial cells. This is initially used to increase the blood compatibility of the adsorbent artificial cells for hemoperfusion (Chang, 1969a). This albumin coating has also been applied to synthetic immnosorbents, resulting in blood compatible synthetic blood group immunosorbents (Chang, 1980d). In addition, Terman et al. (1977) showed in animal studies that albumin-coated collodion activated charcoal (ACAC) can remove antibodies to albumin. This has become a basis of one line of his research in which other types of antigens or antibodies are applied to the collodion coating of the artificial cells to form immunosorbents. Other immunosorbents based on this principle have also been developed for the treatment of human systemic lupus erythematosus; removal of antiHLA antibodies in transplant candidates; treatment of familial hypercholesterolemia with monoclonal antibodies to low-density lipoproteins (Terman, 1980;
Mucopolysaccharides are important components of the surface of cell membranes. Incorporating a polysaccharide, heparin to the artificial cell membrane, increases the biocompatibility and circulation time of artificial cells (Chang et al., 1967, 1968). Incorporation of a synthetic polymer, polyethylene glycol (PEG) into the membrane of artificial cells, nanocapsules and lipid vesicles, is even more effective for increasing biocompatibility and circulation time (Chang et al., 2003; LaVan et al., 2002; Torchilin 2005).

11.7. Drug Delivery

Artificial cell-based drug delivery systems differ from the usual artificial cells discussed up to now. Artificial cells retain the bioreactive material inside them to act on external molecules diffusing into the artificial cells or to release their secretions. For instance, enzyme is retained in an artificial cell to act on the substrate diffusing in while the product diffuses out. Another example is artificial cells containing islet that release insulin. Unlike artificial cells, a drug delivery system separates the drug from the external environment initially, but in order for the system to function, the contents need to be released as and when or where it is needed.

General

This monograph is not about drug delivery, an extremely large and broad area and which are covered in many excellent reviews and books; only the artificial cells related area will be very briefly summarized below. Submicron lipid membrane artificial cells, i.e. lipid vesicles have been developed for sometime now, and are best for use in the delivery of drugs and medications, including chemotherapeutic agents. With the recent interest in biotechnology and gene therapy, there is increasing need for delivery system that can carry sufficient amounts of macromolecules. Thus, biodegradable
STANDARD ARTIFICIAL CELLS: CONTENTS ACT WHILE RETAINED INSIDE AT ALL TIME

Example: enzymes inside act on permeant substrate and releases product
Example: islets inside response to external glucose and secret insulin

ARTIFICIAL CELLS IN DRUG DELIVERY SYSTEMS
Initially retain drugs, hormones, chemotherapeutics, etc inside
BUT
In order to act, have to release contents as and when and where needed

Fig. 11.9. Standard artificial cells compared to extension of artificial cells for use in drug delivery.

Polymeric artificial cells especially those of nano dimensions are of increasing importance. These nanodimension biodegradable polymeric artificial cells are disguised under different names, including nanocapsules, nanoparticles, nanotubules, polymersomes, etc.
**Polymeric semipermeable microcapsules**

Luzzi (1970) used nylon membrane artificial cells, microcapsules, prepared as reported earlier (Chang 1964) to microencapsulate drugs for slow release in oral administration. Others have also extended this approach. However, the modern approaches in drug delivery systems are based on injectable biodegradable systems.

**Biodegradable polymeric artificial cells, nanoparticles, nanocapsules**

Biodegradable membrane artificial cells have been prepared to contain enzymes, hormones, vaccines, and other biologicals (Chang, 1976a). The detailed method is described in Appendix II. The polylactide polymer can degrade in the body into lactic acid and finally into water and carbon dioxide. Variations in preparation (see Appendix II) can result in artificial cells that releases insulin at different rates (Fig. 11.10) (Chang, 1976a).

Biodegradable drug delivery systems are now widely used in different forms ranging from microscopic to nanodimensions. They are also known as nanoparticles, nanocapsules, polymersomes, nanotubules, etc. Langer’ group has written an excellent review on this topic (LaVan et al., 2002). Examples of other references include books and reviews (Malsch, 2005; Gupta and Kompell, 2006; Ranade and Hollinger, 2003; Pardridge, 2002; Duncan, 2003; and many others). As described earlier in this book, this method also forms the basis for preparing nanodimension artificial red blood cells (Chang, 2005, 2006).

**Liposomes evolved into lipid vesicles that are lipid membrane artificial cells**

Bangham first reported the preparation of liposomes each consisting of microspheres of hundreds of concentric lipid bilayers — multi-lamellar (Fig. 11.4) (Bangham et al., 1965). They used these as a membrane model for basic membrane research. Back in the 1960s, I encouraged a
Fig. 11.10. Biodegradable membrane artificial cells have been prepared to contain enzymes, hormones, vaccines, and other biologicals (Chang, 1976a). This figure summarizes the result of polylactide artificial cells prepared using the double emulsion method (Appendix II). Variations in the molecular weight of polylactide and thickness of the membrane can result in artificial cells that release insulin at different rates. Faster release occurs during encapsulation of insulin solution at high concentration, while very slow release occurs during encapsulation of insulin crystals. (Chang, 1976a).

newly graduated McGill Ph.D., Gregoriadis, to look into different ways of forming artificial cells for the delivery of biologics. His subsequent research in England resulted in the first report on the use of liposomes as drug delivery systems that has opened a whole new approach (see 1976 book edited by Gregoriadis). The large amount of lipid in the original multi-lamellar liposome limits the amount of water soluble drugs that can be enclosed. Thus, the basic principle and method of preparing artificial cells using ether as the dispersing phase (Chang 1957, 1964) was extended by researchers into what they called an “ether evaporation method” to form single bilayer (unilamellar) lipid membrane liposomes (Deamer DW and Bangham AD, 1976). These
lipid membrane artificial cells, i.e. lipid vesicles, have since been extensively studied for use as drug delivery systems (Torchilin, 2005).

**Polymer(PEG)-lipid membrane artificial cells or PEG-lipid vesicles**

The original liposome drug carrier consists of a lipid with a water soluble drug entrapped in the aqueous phase, and a water-insoluble drug in the lipid phase. It is good to see that researchers have applied a number of the results of our earlier basic research on surface charge, lipid-polymer membrane, surface modification, and magnetic targeting into the original lipid vesicles for drug delivery. These and other important developments being carried out by many researchers in the field of drug delivery have resulted in important progress in the field. Thus, surface charges are incorporated in liposome for possible targeting of drug and more recently the use of positively charged lipid to complex with DNA. Then a biodegradable polymer, polyethylene glycol (PEG), is incorporated in the liposome surface to result in longer circulation time. Further developments led to the incorporation of antibodies in the lipid membrane to allow for targeting to cells with the corresponding antigens. Magnetic particles loading into liposome allows for magnetic targeting. Thus, lipid vesicles are getting to be more like the lipid-polymer membrane artificial cells (Chang, 1969d, 1972a) and no longer pure lipid vesicles. One major advantage of lipid vesicles is its ability to fuse with cellular membranes or membranes of intracellular organelles. This allows for much versatility in their ability to deliver drugs to different sites of the cells (Fig. 11.11).

As a result of all these developments, and in particular the preparation of polymer(PEG)-lipid membrane artificial cells, there has been much recent progress. A number of drugs in PEG-lipid vesicles has already been approved for clinical use or use in clinical trials. The following are brief examples of some of the drugs and more details are available elsewhere (Torchilin, 2005): “lipid vesicles” containing daunorubicin for Kaposi’s sarcoma; doxurbicin as combination therapy for recurrent breast cancer; doxorubicin for refractory Kaposi’s sarcoma and ovarian cancer and breast cancer; amphotericin B for fungal
Fig. 11.11. Routes of drug delivery of lipid vesicles; these can fuse with the cell membrane to release the drug into the cell. The lipid resides can undergo endocytosis to form endosomes that can enter lysosomes where the drug is released. Thus can also release the drug outside the cell. Lipid vesicles with a surface antibody can bind to the antigen receptor and enter the cell as endosomes.

infections; cytarabine for lymphomatous meningitis; vincristine for non-Hodgkins lymphoma; lurtotecan for ovarian cancer; nystatin for fungal infection; and many others.

**Polymersomes: polymeric membrane artificial cells**

Discher’s group (Discher et al., 1999, Photos et al., 2003) attempted to increase the strength of the PEG-lipid-membrane artificial cells through self-assembling of copolymers to form a membrane of PEG polymer. This significantly increases the circulation time and strength as compared to PEG-lipid membrane artificial cells (Discher et al., 1999; Photos et al., 2003). Thus, liposome has evolved into lipid membrane artificial cells, then polymer(PEG)-lipid membrane artificial
cells and finally back to the original polymeric membrane artificial cells (Chang 1964) disguised under the name of polymersomes.

11.8. Artificial Cells Containing Multienzyme Systems with Recycling of ATP and NADH

Most metabolic functions are carried out in cells by complex multienzyme systems. Thus, artificial cells containing multienzyme systems would have more uses as compared to those containing single enzymes. However, multienzymes require energy from ATP or NAD(P)H and biological cells have the ability to regenerate these for continuous use by the cells. Can we make artificial cells containing useful multienzyme systems that can also regenerate ATP and NAD(P)H?

Artificial cells containing multienzyme system for recycling of ATP

One of the most important cofactor that is recycled in biological cells is ATP. We therefore started by studying artificial cells containing hexokinase and pyruvate kinase for the recycling of ATP (Fig. 11.12) (Campbell and Chang, 1975). Our study showed that this system can effectively recycle ATP as analyzed by the continuous conversion of glucose into glucose-6-phosphate and phosphoenol pyruvate into pyruvate.

Artificial cells containing multienzyme system for recycling of NAD(P)H

Artificial cells containing yeast alcohol dehydrogenase and malate dehydrogenase (Campbell and Chang, 1975, 1976) (Fig. 11.12). NAD$^+$ and NADH can equilibrate rapidly across the membrane to be recycled in the artificial cells. Recycling of NAD$^+$ and NADH follows as shown by the production of malate (Fig. 11.13). When the recycling is blocked, production of malate ceases.
Fig. 11.12. *Upper:* Polymer membrane artificial cells containing hexokinase and pyruvate kinase recyle ATP in the presence of glucose (Campbell and Chang, 1975). Absence of glucose blocks the recyle. *Lower:* Polymer membrane artificial cells containing yeast alcohol dehydrogenase and malate dehydrogenase can recycle NADH as shown by the continuous production of malate (Campbell and Chang, 1975, 1976). In the absence of recyle, malate production ceases.

*Artificial cells containing urease, glutamate dehydrogenase and glucose-6-phosphate dehydrogenase*

Since the needed NADH can be recycled, the next study was to see whether we could use a multienzyme system for the removal of urea. First, we used a model system of polymer membrane artificial cells loaded with three enzymes: urease, glutamate dehydrogenase and glucose-6-phosphate dehydrogenase (Fig. 11.13) (Cousineau and Chang, 1977; Chang and Malouf, 1979; Chang et al., 1979b). Urease converts urea into ammonia. Then glutamate dehydrogenase converts...
Fig. 11.13. Polymer membrane artificial cells containing urease, glutamate dehydrogenase, glucose-6-phosphate dehydrogenase for the multistep enzyme reaction of conversion of urea into amino acid glutamic acid and for the recyling of NADPH. Urea is lowered without any increase in ammonia level, as ammonia is converted into glutamate (Cousineau and Chang, 1977; Chang and Malouf, 1979; Chang et al., 1979b).

ammonia into glutamic acid. Glucose-6-P dehydrogenase serves to recycle of NADPH.

The rate of conversion of urea into glutamate and the recycling of NADPH could be followed by the rate of formation of 6-phosphogluconate, decrease in urea level, or the formation of glutamic acid. Ammonium acetate could be used in this reaction instead of urea. Figure 11.13 shows that the area level is lowered without any increase in ammonia level, as ammonia is efficiently converted into glutamate. Glutamate is formed at the same rate irrespective of whether urea or ammonium acetate is used as the substrate. This suggests that the rate-limiting step in this sequential reaction is in the conversion of ammonia into glutamic acid.

**Dextran-NADH retained and recycled inside artificial cells (Fig. 11.4)**

The above studies show that artificial cells containing multienzyme systems can carry out sequential enzymatic reactions to convert urea into glutamate and at the same time recycle the required NADH. The above study is a model experiment to test this feasibility. However, in potential medical or engineering uses, one would prefer to have the cofactor retained and recycled inside the artificial cells. This way, it
will not be necessary to have NADH supplied from outside. The first
step is to see how we can retain the NADH inside the artificial cells
without decreasing the permeability to other substrates and products.
We use Mosbach’s method (Larsson and Mosbach, 1971) to covalently
link NADH to a macromolecule to form a soluble NADH-dextran.
We found that the resulting NADH-dextran can be retained inside the
artificial cells where it is recycled (Fig. 11.14) (Campbell and Chang,

The artificial cells are retained in a flow reactor perfused by the
substrates. The recycling activity is followed by the production of
malate (Table 11.2). The recycling activity of the shunt is high as
65% of the oxaloacetic acid is converted into malic acid in one
passage through the shunt. The dextran-NAD\(^+\) present within the

\[
\begin{align*}
&\text{ethanol} \rightarrow \text{YADH} \rightarrow \text{dextran-NAD}^+ \rightarrow \text{MDH} \rightarrow \text{malate} \\
&\text{dextran-NADH} \rightarrow \text{acetaldehyde} \rightarrow \text{malate} \rightarrow \text{oxaloacetate}
\end{align*}
\]

Fig. 11.14 and Table 11.2. Artificial cells containing alcohol dehydrogenase,
malate dehydrogenase and soluble NADH-dextran. NADH-dextran is retained
inside the artificial cells where it is recycled (Campbell and Chang, 1978;
formed using Mosbach’s method (Larsson and Mosbach, 1971). Table 11.2
shows the rates of regeneration of NAD\(^+\).

<table>
<thead>
<tr>
<th>Storage (days)</th>
<th>NAD(^+) Regenerated (cycles/h)</th>
<th>Amount in Artificial Cells (μmole/h/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>120.0</td>
<td>150.0</td>
</tr>
<tr>
<td>1</td>
<td>117.5</td>
<td>147.0</td>
</tr>
<tr>
<td>4</td>
<td>84.0</td>
<td>105.0</td>
</tr>
<tr>
<td>7</td>
<td>37.3</td>
<td>46.5</td>
</tr>
</tbody>
</table>
Artificial Cells

microcapsules is regenerated two times each minute during the reaction. The shunt showed good stability, with the reaction rate remaining constant for the first hour and 83% of the original activity being retained even after 3 h of continuous reaction. The same shunt could be stored and reused several times. It is important to use purified hemoglobin instead of crude hemoglobin for coencapsulation. Crude hemoglobin contains impurities that interfere with the recycling.

**Multienzyme with NAD-dextran for conversion of waste, urea, into useful essential amino acids**

We first showed that artificial cells containing multienzyme systems and NAD$^+$-dextran can convert urea or ammonia into glutamate (Gu and Chang, 1988b, 1988c). The next step was to see if we could use a more complex multienzyme system and NAD$^+$-dextran to convert the waste, urea, into useful products, essential L-branched-chain amino acids, L-leucine, L-valine and L-isoleucine. This is based on artificial cells containing urease to convert urea into ammonia and leucine dehydrogenase to convert the ammonia formed into essential amino acids. Glucose dehydrogenase is also included for the recycling of NAD$^+$-dextran using glucose as source of energy for cofactor recycling (Gu and Chang, 1988a). Other enzymes for recycling of NAD$^+$-dextran include yeast alcohol dehydrogenase that uses alcohol as source of energy (Gu and Chang, 1990a); malate dehydrogenase that uses malate as a source of energy (Gu and Chang, 1990b) and L-lactic dehydrogenase that uses L-lactic acid as a source of energy (Gu and Chang, 1991). The results obtained using artificial cells containing leucine dehydrogenase, urease, glucose dehydrogenase and NAD$^+$-dextran is shown below (Fig. 11.15) to illustrate this approach.

**Recycling of free NADH retained within the lipid-polymer membrane artificial cells for conversion of urea into amino acid**

We have studied another way to retain NADH inside artificial cells without crosslinking it to dextran or other macromolecules. The
Perspectives on the Future of Artificial Cells as Suggested by Past Research

Fig. 11.15. Upper: Artificial cells containing urease, leucine dehydrogenase, glucose dehydrogenase, dextran-NADP⁺ to convert the waste, urea, into useful essential L-branched-chain amino acids, L-leucine, L-valine and L-isoleucine. Glucose dehydrogenase, uses glucose as a source of energy to recycle NAD⁺-dextran (Gu and Chang, 1988a). Lower: The effect of D-glucose concentrations on the production rates of L-leucine, L-valine and L-isoleucine. The production rates for 200mM D-glucose are used as the 100% control.
membrane of biological cells can retain free NADH without the need to be crosslinked to macromolecules. Thus, use our lipid-polymer membrane artificial cell (Chang, 1969d, 1972a) that is only permeable to ammonia and urea but not to larger molecules like NADH. Biological cell membranes have transport mechanism to transport glucose that cannot diffuse across the cell membranes. Since we do not yet have a glucose transport carrier in the lipid-polymer member, we have to replace glucose dehydrogenase with alcohol dehydrogenase. This way, external ethanol can enter the artificial cells and serve as a source of energy for the recycling of NADH (Fig. 11.16) (Yu and Chang, 1981a, 1981b, 1982a, 1982b; Ilan & Chang, 1986).

The standard lipid-polymer complexed artificial cell membrane is not permeable to urea (Table 11.3). By decreasing the proportion of cholesterol in the lecithin-cholesterol component, the membrane becomes permeable to urea (Table 11.3) but with no leakage of NAD$^+$ and ketoglutarate.

**Fig. 11.16 and Table 11.3.** Recycling of free NADH retained within the lipid-polymer membrane artificial cells for the conversion of urea and ammonia into glutamate. The lipid-polymer membrane artificial cells contain three enzymes, urease, glutamate dehydrogenase and alcohol dehydrogenase. Table 11.3 shows the effects of permeability to urea and rate of conversion of urea into glutamic acid (Yu and Chang, 1981a, 1981b, 1982a, 1982b, Ilan and Chang, 1986).
Table 11.3 Multienzyme Lipid-Polymer Membrane Artificial Cells Convert Urea into Glutamate with NADH Recycling

<table>
<thead>
<tr>
<th>Permeability to Urea</th>
<th>Glutamic Acid (μmol/L/3h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low</td>
<td>0</td>
</tr>
<tr>
<td>Medium</td>
<td>2.42</td>
</tr>
<tr>
<td>High</td>
<td>6.93</td>
</tr>
</tbody>
</table>

Summary

The above basic research shows that it is possible to prepare artificial cells containing multistep enzyme systems with recycling of ATP and NAD(P)H. Further developments may lead to a number of possible applications. One example is to complete the urea removal component of the miniaturized hemoperfusion-ultrafiltration system for treating kidney failure. We have already applied this basic information in the preparation of nano artificial red blood cells that contain multienzyme system with cofactor recycling to convert methemoglobin back to hemoglobin (Chang, 2005i, 2006a). Potentially, any combinations of enzyme systems can be enclosed within each artificial cell. The lipid-polyamide membrane artificial cell can also be used in basic research as a cell model.

11.9. Artificial Cells Containing Microsomes, Cytosol Ribosomes and Polymerases

Introduction

Chapter 5 shows that it is possible to prepare complete artificial red blood cells containing hemoglobin and all the complex enzyme systems of its biological counterpart. In addition, safety and efficacy in animal studies have also been shown. The next step is to prepare artificial cells that have the properties of more complex biological cells.
Artificial cells containing liver microsomes and cytosol

We isolated microsomes and cytosol from rat liver and encapsulated these into cellulose nitrate membrane artificial cells (Yuan and Chang, 1986). The method is based on the standard method (Chang, 1964, 1972a), with the updated method (Chang, 2005a) available in Appendix II. NADPH-cytochrome C reductase and lactate dehydrogenate are used as the marker enzymes for, respectively, microsomes and cytosol. Table 11.4 shows that after encapsulation into artificial cells, NADPH-cytochrome C reductase, marker enzyme for microsomes, retained 24.5% of its original activity. Lactate dehydrogenase, enzyme marker for cytosol, retained 11% of its original activity after microencapsulation into artificial cells. It is important to carry out the preparation at 4°C instead of 23°C.

The result shows that artificial cells can be used to encapsulate subcellular organelles. Liver cells contain lysomes which when released can destroy the subcellular organelles. By preparing the homogenate of the liver cells and rapidly removing the lysosome fraction, we can reconstitute the other organelles and cytosol into an artificial cell free from the possible proteolytic effects of the lysosomes.

Table 11.4 Artificial Cells Containing Liver Microsomes and Cytosol

<table>
<thead>
<tr>
<th>NADPH- Cytochrome C Reductase as Marker for Microsomes</th>
<th>Lactate Dehydrogenase as Marker for Cytosol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Original Activity (units)</td>
<td>Activity Recovered (units)</td>
</tr>
<tr>
<td>---------------------------</td>
<td>---------------------------</td>
</tr>
<tr>
<td>Microsome enzyme marker</td>
<td>8550</td>
</tr>
<tr>
<td>Cytosol enzyme marker</td>
<td>2150</td>
</tr>
</tbody>
</table>

Towards a “living” artificial cell containing polymerases, ribosomes and transcription/translation system

An even more exciting and ambitious effort is the recent serious attempts to develop a “living” artificial cell. A “living” artificial cell is
defined by Deamer (2005) as an artificial cell that can capture energy, maintain ion gradients and contain macromolecules. Macromolecules must be able to grow by polymerization and evolve in a way that speeds up growth. They must be able to store information and possess the ability to mutate and direct the growth of catalytic polymers. They must also be able to reproduce themselves and divide. Studies on artificial cells described earlier in this monograph suggest that many of these criteria can be met. However, preparing artificial cells that can reproduce themselves and divide will be extremely difficult. Some of the exciting studies on “living” artificial cells are highlighted in the next paragraph.

Szostak, at Harvard, have been carrying out a number of studies (Hanczyc et al., 2003; Chen et al., 2004; Josephson et al., 2005). For example, they have prepared a simplified model artificial cell by putting simple RNA enzymes inside the cells, and showing that the cells can conduct their characteristic activities. They are also carrying out the modification of the cellular translational system to allow for the ribosomal translation of molecules for medical uses. Monnard and Deamer (2001) prepared models for primitive cellular life by encapsulating T7 RNA polymerases and templates into lipid membrane artificial cells. This enzyme was able to synthesize an RNA transcript from the DNA template that required four nucleoside triphosphates. Since the lipid membrane was not permeable to nucleoside triphosphates, they had to use DMPC liposomes at a temperature of 23°C. Despite this, permeation was still very slow, thus limiting the rate of RNA synthesis. Other researchers encapsulated a complex polymerase system into lipid membrane artificial cells and showed that the PCR reaction could be carried out (Oberholzer et al., 1995). Since the substrate could not diffuse across the lipid membrane, it had to be loaded together with the whole system. The result was that there was only enough substrate to produce a very small amount of the product. They have also encapsulated ribosomes into lipid membrane artificial cells and obtained some translation product. Noireaux and Libchaber (2004) from Rockefeller University were able to engineer a DNA plasmid to express proteins and encapsulate this into lipid membrane artificial cells. However, they could only produce proteins
Artificial Cells

for a few hours because the source material inside the artificial cells could not be replaced. They solved this problem by incorporating a channel-forming protein, alpha hemolysin, into the lipid artificial cells membrane. This way, the artificial membrane could become permeable to larger molecules like nucleotides. As a result, they could function for up to four days. The use of selectively permeable crosslinked protein membrane or polymeric membrane artificial cells as described in Section 11.3 above (Chang, 1964, 1972a, 2005i) can result in an even more efficient supply of external substrates.

In another study, Griffiths and Tawfik (2000) used compartmentalization to load the transcription/translation system into a water-in-oil emulsion. This way, each gene could occupy a separate water emulsion to carry out its function. A more efficient approach may be possible by using the polymeric compartmental system of artificial cell where each artificial cell contained “subcellular compartments” with ultrathin polymeric membranes (Chang 1965, 1972a; Chang et al., 1966, 2005i) (Fig. 2.4). This way, biologically active systems could be enclosed separately or in combination into each of these artificial subcellular compartments. Since the permeability of each of the compartments could be individually and separately adjusted, this might allow for more efficient stepwise functions.

The most difficult steps will be attempts to prepare artificial cells that can reproduce themselves and divide. To do this, the artificial cells have to contain genes and enzymes that can be replicated and shared among daughter cells. One possible way is to use a self-replicating ribozyme that can duplicate itself and act as both genetic material and the catalyst for replication. Johnston et al. (2001) used a ribozyme to catalyze the replication of another RNA template molecule. Their next step is to verify that the RNA can continue with the process of replication. Rasmussen et al. (2003) of Los Alamos National Laboratory used computer simulation to design a micelle with information coding and metabolic machinery on its exterior. They used DNA-mimics conjugated to a light-sensitive molecule. In theory, light would trigger the conversion of substrates into peptide nucleic acid (PNA) based on the PNA template. These are incorporated into the micelle that would grow and divide. The next step is to test this theoretical simulation
experimentally. Ishikawa et al. (2004) of the University of Osaka, Japan used a lipid membrane artificial cell containing a bacterial plasmid and SP6 RNA polymerase, which acted as a driving force for the production of a T7 RNA polymerase needed to catalyze the transcription of a gene. Thus, this membrane-bound system shows both gene expression and a two-stage genetic cascade of events. Information regarding the minimal number of genes needed for a “living” artificial cell is important. Kobayashi et al. (2003) identified the minimum set of genes in *Bacillus subtilis*, narrowing the number to 271 and only 4% of these have unknown functions. It would help if this number can be narrowed down even further to allow for the preparation of a primitive “living” artificial cell.

All these exciting and ambitious efforts show that many of the criteria for a “living” artificial cell can possibly be met. The most difficult criterion is the ability of the “living” artificial cell to reproduce itself and divide. Instead of using lipid membrane artificial cells alone, a combination with crosslinked-protein membrane or polymeric membrane artificial cells could be used — either individually or in combination.

11.10. New Generations of Computer System and Nanoscale Robotics Based on Artificial Cells

Researchers in this field suggest that next generation self-repairing computer and robotics technology requires the use of intelligent technical systems based on artificial cells. Toward this end, the European Commission is supporting an integrated program of “PROGRAMMABLE ARTIFICIAL CELL EVOLUTION” (PACE) (http://wills.gex.gmd.de/bmcmyp/Data/PACE/Public/). This is aimed at a new generation of embedded information technology using programmable, self-assembling artificial cells. To achieve this, the PACE project focusses on the intelligent technical (IT) potential of artificial cells that are truly “artificial” and not replicates of biological cells. This project consists of a consortium of 13 partners and two cooperating groups from eight European countries and the USA. The group includes expertise in complex systems, embedded systems,
robotics, evolution, statistics, chemical kinetics, physical simulation, microfluidics, organic and bioorganic chemistry, computer interfaces and control systems. With this interdisciplinary approach, they plan to prepare microscopic artificial cells as chemical information processing systems. They suggest that for the next-generation robotics and nanoscale technologies to be realized, they would need distributed processing systems with self-organizing properties in the form of artificial cells. These will be designed and assembled automatically from non-living materials.

They have developed a computer-programmable closed-loop controller, based on microfluidic technology. This can be used to observe and control the local physical environment of cell-sized chemical subsystems. They have also shown that an artificial peptide nucleic acid (PNA) can synthesize or direct the information in the synthesis of another copy of itself — a step towards self-replication. They have also developed simulation models to describe artificial cells on an intermediate physical level to follow the control, stability and breakdown of the subsystems. They are also studying the use of artificial cells as a novel platform for self-assembling microscopic robots, both in simulation and experiments. On an ethical level, they emphasize that “What we are talking about is not chemically making a copy of an existing artificial cell, but chemically synthesizing something which we could call an artificial cell.”

11.11. The Future of Artificial Cells

Nature’s creations are complex, intricate and ingenious. For ages, humans have tried to reproduce exact replicates of some of these without success. Success only comes to those who are humble enough to use the principles learned from nature to produce much simpler systems instead of trying to replicate nature.

A good example is in the many unsuccessful and often fatal attempts made by humans to fly like birds. The first initial success is when the Chinese, instead of trying to fly like birds, use the principle to build very simple kites that later can even carry humans. These evolve into gliders. Since then, each major progress in flight is related to major
progresses in other areas. Thus, the invention of the motor engine in Europe allows the gliders to evolve into the first propeller driven planes first in the US and then Europe. Centuries ago, the Chinese invented fireworks and rocket-propelled weapons. This principle has been applied to jet engine that allows the propeller planes to evolve into the first jet-planes in Germany. These have now reached enormous size and speed. Further developments allows human to travel to space first in Russia and then the USA. This is an example of how progress is made on both an interdisciplinary and international basis. Much delay can be the result if there is not enough interaction between the different disciplines and countries.

For many years, scientists have carried out tremendous amounts of research on the structural and functional basis of biological cells. However, attempts to reproduce exact replicas of biological cells have not been successful. On the other hand, the purpose of the very first humble “artificial cells” (Chang, 1957) was not to reproduce biological cells, but to use available basic knowledge to prepare very simple systems for possible uses in medicine and other areas. Each major progress in other areas has led to stepwise progress in artificial cells. First, there was the coming of age of polymer chemistry and biomaterials. Then there was the recognition of the importance and developments in biotechnology. There is the present ongoing progress in molecular biology and genomics that will contribute to a quantum leap in the area of artificial cells, including the recent efforts on “living” artificial cells. One can expect that there will be important future progress in other areas that will contribute to unlimited progress in the area of artificial cells. One example is in the use of artificial cells for next generation computer and nanoscale robotics.

The following prediction in my 1972 monograph on “Artificial Cells” is already out of date: “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”. In the last 50 years (Chang, 1957), artificial cells have progressed way beyond this 1972 prediction. Artificial cells can now be of macro, micro, nano and molecular dimensions.
There are also unlimited possibilities in variations for the artificial cell membranes and contents. Searching for “artificial cells” on the Internet (for example, www.google.com) gives more than 50,000 hits in all areas of artificial cells. Even then, we have only just touched the surface of the enormous potential of artificial cells. One hopes that the many arbitrary subdivisions of “artificial cells” under the guise of different names can be brought together! When this takes place, the result of the pooling of talents, specialized know-how in this very interdisciplinary and international area will lead to progress beyond anyone’s imagination.