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Microfabricated biocapsules for the immunoisolation of pancreatic islets of langerhans

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Microfabricated Biocapsules for the Immunoisolation of Pancreatic Islets of Langerhans

by

Tejal Ashwin Desai

DISSERTATION

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This dissertation is dedicated to my parents, Ashwin and Surekha Desai, and my brother Amit, who have always believed in me and have forever encouraged me to pursue my goals to the end.

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I have been extremely fortunate to have amazing mentors and role models in my life. They have inspired me to pursue my interests in bioengineering teaching and research, yet maintain my strong commitment to community service and leadership. They have continued to challenge and encourage me through my educational journey, despite my moments of doubt.

In particular, I thank my advisor, Professor Mauro Ferrari. I am grateful for the rare opportunity to work with an individual who dares to be innovative, creative, and challenges his students to push the boundaries of disciplines to new frontiers. I am inspired by the perseverance and strength he has displayed over the last four years and I can only hope that I will inspire students as he has. His friendship and support is something I will always treasure.

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Finally, thank you to my family near and far. Your words of encouragement have been invaluable in helping me achieve my dreams.

Abstract

Microfabricated Biocapsules for the Immunoisolation
of Pancreatic Islets of Langerhans

by

Tejal Ashwin Desai

Doctor of Philosophy in
Engineering Science - Bioengineering
University of California, San Francisco and Berkeley
Professor Mauro Ferrari, Chair

A silicon-based microfabricated biocapsule was developed and evaluated for use in the immunoisolation of transplanted cells, specifically pancreatic islets of Langerhans for the treatment of Type I diabetes. The transplantation of cells with specific functions is a promising therapy for a wide variety of pathologies including diabetes, Parkinson's, and hemophilia. Such transplanted cells, however, are sensitive to both cellular and humoral immune rejection as well as damage by autoimmune activity, without chronic immunosuppression. The research presented in this dissertation investigated whether microfabricated silicon-based biocapsules, with uniform membrane pore sizes in the tens of nanometer range, could provide an immunoprotective environment for pancreatic islets and other insulin-secreting cell lines, while maintaining cell viability and functionality.

By utilizing fabrication techniques commonly employed in the microelectronics industry (MEMS), membranes were fabricated with precisely controlled and uniform pore sizes, allowing the optimization of biocapsule membrane parameters for the encapsulation of specific hormone-secreting cell types. The biocapsule-forming process employed bulk micromachining to define cell-containing chambers within single crystalline silicon wafers. These chambers interface with the surrounding biological environment through polycrystalline silicon filter membranes, which were surface micromachined to

present a high density of uniform pores to allow sufficient permeability to oxygen, glucose, and insulin. Both in vitro and in vivo experiments established the biocompatibility of the microfabricated biocapsule, and demonstrated that encapsulated cells could live and function normally in terms of insulin-secretion within microfabricated environments for extended periods of time. This novel research shows the potential of using microfabricated biocapsules for the encapsulation of several different cell xenografts. The semipermeability of microfabricated biocapsules, their biocompatibility, along with their thermal and chemical stability, may provide an improved encapsulation device for the immunoisolation of cell xenografts in hormone-replacement and cell-based therapies.

Professor Mauro Ferrari, Chair

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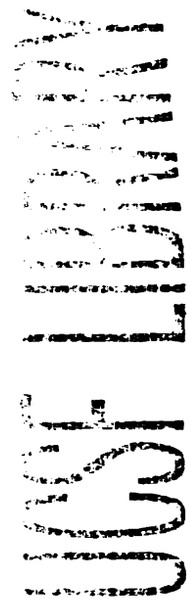
1. Introduction and Overview

1.1 Biomaterials in Bioengineering

As we approach the end of the millennium, the promise of biomedical technology has become a reality. Biomaterial-containing implants ranging from synthetic vascular grafts to artificial joints and drug delivery systems have become commonplace. Each year there are over 20,000 transplants and over 2,000,000 biomaterial implants [NIH Report, 1997]. These numbers illustrate the need to continue aggressive research and development of improved biomedical devices and biomaterials for therapeutic use.

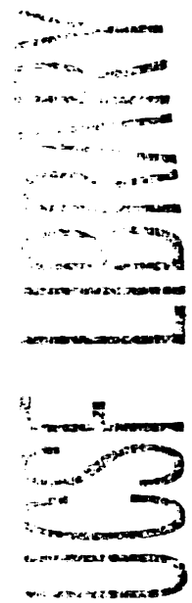


More specifically, there is a need to begin investigations on the next generation of medical implants and biomaterials that are safe, reliable, functional, and long-lasting. An integrated and multidisciplinary research approach is essential in order to advance our understanding of biological systems and begin to design and develop novel synthetic medical materials that are compatible with the environment of the host and significantly increase the functional lifetime of implants.

The types of biomaterials used for implants have been limited by the extent to which they are deemed biocompatible and biofunctional. Thus, the key to the future of biomedical implants lies in the development of materials with improved longevity and functionality. This challenge must be approached from several directions, incorporating the perspectives and knowledge of such individuals as the material scientist, the biologist, the

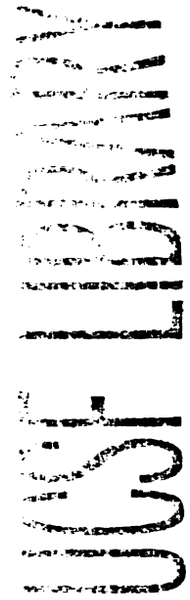
chemical engineer, the pathologist, and the clinician. Only then can one design, fabricate, and test biohybrid devices that will meet the goals of a particular biomedical application.

Integrating the diverse fields of biotechnology, medicine, and material science has led to an emergence of a new class of therapeutic biomaterial devices which incorporate living cells with synthetic components for cellular biosynthesis and hormone secretion. Such devices are able to replace or supplement a deficient cell-secreted product by completely replacing the patient's diseased or destroyed cells with new cells encapsulated within a synthetic protective "shell." Although many of the materials and devices currently in use are functionally adequate for short term applications, they are not optimal and can be associated with chronic complications such as poor biocompatibility, biodegradation, calcification, cellular disintegration and varying degrees of immune responses [Hellman et al., 1994]. In particular, the development of encapsulated cellular implants requires the careful study and optimization of three main components: the capsule and membrane material, the intracapsular microenvironment, and the cells themselves. This dissertation will attempt to address these components in relation to the new technology proposed, that of a microfabricated biocapsule for the therapy of diabetes. It is expected that the technology can also be applied to the treatment of other diseases resulting from hormonal deficiencies.



1.2 *BioMEMS for Cellular Therapeutics*

Research on microfabricated devices for biomedical applications (BioMEMS) has rapidly advanced in the last few years. The suitability of such devices for laboratory and clinical application has been widely documented with extensive research and development efforts in large research universities as well as in the industrial sector. While the majority of BioMEMS research has focused on the development of diagnostic tools such as electrophoretic, chromatographic, biosensor, and cell manipulation systems[Baxter, Bousse, et al., 1994; Mathies, 1992; Brody and Yager, 1996], relatively few researchers have concentrated on developing microfabricated devices for therapeutic applications, including neural regeneration, CNS stimulation, and microsurgery [Edell, 1992; Kovacs et al., 1994; Wise and Najafi, 1991]. In this thesis, a microdevice for the immunoisolation of cell xenografts is presented, which involves the use of microfabrication technology to produce a silicon-based implantable biocapsule.



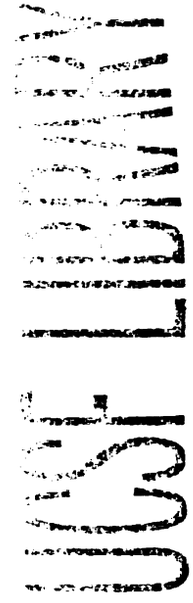
The concept of a biocapsule emerged over thirty years ago as a possible way in which transplanted tissue could be protected from immune rejection by enclosure within a semipermeable membrane[Lim and Sun, 1980]. The transplantation of certain cells has been reported to be a promising direction for the therapy of several pathologies, such as Type I Diabetes, Alzheimer's, Parkinson's, and hemophilia [Colton, 1995]. Most often, cellular transplantation involves isolation of primary cells such as pancreatic islets of Langerhans or hepatocytes. It has been demonstrated that these primary

cellular transplants can respond physiologically both in vitro and in vivo by secreting bioactive substances in response to appropriate stimuli, as long as they are sufficiently immunoprotected. The approach of cell encapsulation has the potential to allow not only allogeneic transplantation without immunosuppression, but also permit the use of xenogeneic tissue sources. Due to the shortage of human donors, the use of xenografts is especially critical for therapies that primarily rely on organ transplantation.

Additionally, an alternative to the encapsulation of naturally occurring primary cells is the encapsulation of tumor cell lines, such as insulinomas, or genetically altered "universal" cell lines such as fibroblasts or myoblasts. Research on tumor or engineered cell lines indicates greater longevity relative to natural cell lines, possibly leading to a prolonged in vivo life of the implant. The use of such cells in encapsulation devices may be an additional means to improve or extend biocapsule performance.

The research presented here has focused on developing an immunoisolating microfabricated biocapsule for the transplantation of pancreatic islets of Langerhans as a therapy for diabetes. It represents the first time microfabricated devices have been investigated for use in cellular therapeutics in general, and specifically for diabetes. Since diabetes and its complications are the third leading cause of death in the United States [Colton and Avgoustiniatos, 1991], there is still an urgent need to find treatment alternatives, despite the recent development of better oral and injectable pharmaceuticals.

Type I Diabetes is a chronic autoimmune disease in which antibodies and/or lymphocytes selectively destroy the insulin-secreting pancreatic islets of Langerhans, resulting in hyperglycemia. Thus, the affected individuals are dependent on exogenous insulin for life. Daily injections of insulin, however, do not necessarily result in normal levels of blood glucose. The inadequacy of conventional insulin-therapy for the treatment of Type I Diabetes has stimulated research on several therapeutic alternatives. One of the most physiological alternatives to insulin injections is the transplantation of the pancreatic islets of Langerhans. The beta cells of these islets that secrete insulin in response to increasing blood glucose concentrations. Ideally, transplantation of pancreatic islet cells (allografts or xenografts) could restore normoglycemia. However, as with most tissue or cellular transplants, the islet grafts, particularly xenografts, are subjected to immunorejection in the absence of chronic immunosuppression.

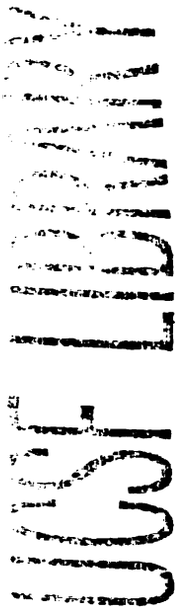


To overcome the need for toxic immunosuppressive drugs, the concept of isolating the islets from the recipient's immune system within biocompatible semipermeable capsules was developed [Lim and Sun, 1980; Lanza et al., 1992; Scharp et al., 1984; O'Shea et al., 1984]. In principle, the biocapsules allow for the free diffusion of glucose, insulin, and other essential nutrients for the islets, while inhibiting the passage of larger entities such as antibodies and complement components [Goosen et al., 1985]. This selective permeability can allow for the physiological functioning of the islets, while preventing acute and chronic immunorejection.

The requirements for an immunoisolating biocapsule are numerous. In addition to well-controlled pore size, the capsule must exhibit stability, non-biodegradability, and biocompatibility. All encapsulation methods to date have used polymeric semipermeable membranes[Weber et al., 1991; Calafiore et al., 1990; Lafferty, 1989; Sugamori, 1989]. However, these membranes have exhibited insufficient resistance to organic solvents, inadequate mechanical strength, and broad pore size distributions --all of which eventually lead to destruction of cell xenografts[Lacy et al., 1992; Ross, 1993]. The most common method of immunoisolation, that of polymeric microcapsules, have the disadvantages of limited retrievability (most often due to biodegradation or fibrous encapsulation) and possible mechanical failure of the spherical membrane. These characteristics limit the use of microcapsules for non-immunosuppressed xenotransplantation [Colton, 1995]. These limitations, in conjunction with deficiencies in transport requirements, have resulted in minimal clinical success of encapsulated islet transplantation, with less than 30 documented cases of insulin independence occurring from over 250 attempts at clinical islet allo-transplantation since 1983 [Lanza and Chick, 1995].

The technology involved in the silicon-based microfabricated biocapsule is based on the creation of a membrane with absolute and uniform pore sizes in order to provide better immunoisolation and physiologic control of cellular transplants. By taking advantage of silicon bulk and surface material properties, membrane structures can be precisely engineered to perform specific functions. The microfabricated biocapsules, due to their material

properties, can provide the advantages of mechanical stability, uniform pore size distribution, and chemical inertness. Thus, microfabrication technology may be advantageous in the field of tissue engineering by creating controlled microenvironments to stimulate and enhance transplanted cell behavior. This technology brings together synthetic and non-synthetic components in order to replace lost or damaged tissue in a host. It has been widely shown that isolated cultured cells grow and organize in contact with synthetic materials which serve as scaffolds for the cells[Brendel et al., 1994]. Therefore, silicon microfabrication of three-dimensional microenvironments for cells opens up many new possibilities in terms of tissue engineering and cell transplantation.



1.3 Research Goals

The goal of this research was to realize a prototypical biocapsule embodying several design considerations, to test *in vitro* its suitability as a biocompatible capsule to house pancreatic islets of Langerhans, and to ultimately determine whether this device could be implanted *in vivo* and maintain some degree of cell viability and immunoprotection. It is hoped that this research will ultimately lead to new and improved therapeutic technologies for the treatment of a wide variety of chronic diseases.

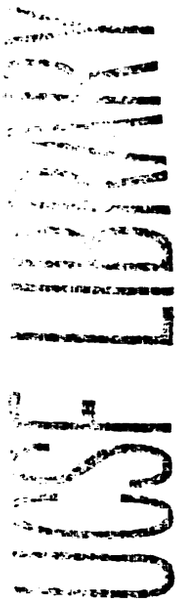
The dissertation is divided into three main sections. Part I focuses on materials for bioengineering, from both a biologic and material science perspective. This section serves to give an overview of desirable biomaterial properties as well as a review of the technology involved in this project.

Section 1 presents an overview of traditional biomaterial applications, the current status of biomaterials and devices, and direction towards which the field is heading. Sections 2 and 3 then describe characteristics necessary in design of a successful biomedical implant, starting from the bulk material and ending with specific surface properties which affect biocompatibility. Finally, section 4 describes in detail that history and background of microfabrication technology, with focus on its use in biomedical technology. An overview of silicon micromachining and processing will be presented.

Part Two focuses on understanding the concepts associated with immunoisolative cellular therapy. Section 5 begins with an overview of transplantation immunology and moves into the specific application of this immunoisolation for Type I Diabetes Mellitus. Section 6 focuses on the fundamental physiology and cell biology needed for understanding disease progression and the development of cell encapsulation therapy. Finally, sections 7 and 8 concentrate on the engineering principles involved in designing and fabricating a cell encapsulation system, including issues of processing, biocompatibility, and transport. A discussion of previous methods and materials used for cell immunoisolation is included as well as the limitations associated with these approaches.

Part III examines the microfabricated biocapsule, a new application of microfabrication technology which is introduced and studied in depth in this research. Starting with fabrication designs and techniques, Section 9 concludes with descriptions of the prototypical microfabricated biocapsule and a

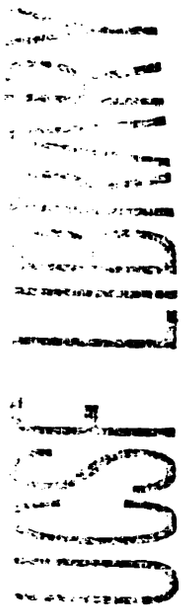
discussion of assembly and packaging issues. The majority of Part III describes biological testing of the microfabricated biocapsule both in vitro and in vivo. Section 10 evaluated issues of biocompatibility and cell growth of the biocapsule material, as well as strategies to improve surface properties. Section 11 then moves into testing of the functionality and performance of the biocapsule in terms of maintenance of cell function with biocapsular environments in vitro. Section 12 continues the discussion of cell functionality by examining the immunoisolation parameters of the biocapsule. Section 13, an examination of the microfabricated biocapsule in vivo, is the culmination of biological testing and concludes with a discussion on the demonstrated potential of using this technology for cellular therapeutics.



2. Biomaterials

2.1 General Properties of Biomaterials

The development of biocompatible biomaterials has become increasingly important for today's emerging medical technology. All biomaterial applications involve at least one interface between a biological and material system. It is likely that biomolecules continuously adsorb and desorb at the interface throughout the lifetime of the implanted biomaterial. The biocompatibility of this material depends on the role of interfacial proteins and cellular recognition processes which occur at the interface. Therefore, one must examine the intrinsic properties of surfaces that direct specific biological responses in order to develop better biomaterials. Surface characterization is extremely critical in biomaterials science because of this correlation between surface composition and biocompatibility.



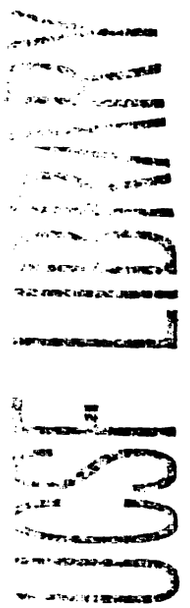
2.1.1 Physical environment of biomaterials

Whenever a foreign material is introduced into living tissue, interfaces are created between the material and the surrounding tissue, be it soft tissue (i.e. blood vessels or skin) or hard (i.e. bone or cartilage). The biologic environment consists of water, oxygen, positive and negative ions, proteins and other biomolecules and cells. The material surface, consisting of individual atoms, molecules, or larger polymeric structures, can interact to form complex interfaces which influence their surrounding environment. Because the surface can change in response to the environment, it is difficult to make accurate assessments of the

surface. It is important to realize that the environment in which the biomaterial is being studied may directly affect the properties of the biomaterial of interest.

Table 2.1 Physicochemical and Mechanical Conditions of Humans [Adapted from Black, 1981]

	Value	Location
pH	6.8	Intracellular
	7.0	Interstitial
	7.15-7.35	Blood
pO_2	2-40	Interstitial (mm Hg)
	40	Venous
	100	Arterial
Temperature	37	Normal Core
	28	Normal Skin
Mechanical Stress	$4x10^7$ N m^{-2}	Muscle (peak stress)
	$4x10^8$ N m^{-2}	Tendon (peak stress)
Stress Cycles (per year)	$3x10^5$	Peristalsis
	$5x10^6$ - $4x10^7$	Heart muscle contraction



The surface of a biomaterial must exist in a unique environment, that of the human body. This means it must maintain stability for long periods of time in the body's warm (37°C), humid, and corrosive environment. The chemical constituents and pH of the biological environment also strongly influence interactions at the biomaterial interface. For example, a metal may degrade by corrosion, releasing metal ions from the surface into solution. These ions then may migrate through the biological system, causing systemic damage to the patient [Kasemo and Lausmaa, 1994].

In a reactive environment such as the human body, free surfaces will react almost instantly to form new bonds and compounds. This is why the surface of a biomaterial usually differs significantly in structure and chemistry from that of

the bulk of the material. These differences can come about through surface contamination, molecular orientation, and surface reactions [Ratner, 1982]. However, in all cases, the driving force for these differences is the tendency for the material to reduce its interfacial energy.

High energy surfaces such as those of metals and inorganic materials have a large driving force to reduce their interfacial energy. Thus, these surfaces will become contaminated with a monolayer of organic material almost instantaneously, as long as the entire energy of the system is lowered. On the other hand, polymeric materials have a much lower surface free energy and thus, their tendency to become contaminated is much lower.

Similar to surface contamination, the driving force for surface molecular reorientation is also the reduction of surface free energy. In polymeric systems, chain segments or functional groups can move to and from the surface. For example, in block copolymers, low energy blocks will tend to migrate towards the surface. In addition, polar side groups or nonpolar backbones can respond to their environment by orienting themselves in a particular way at the interface.

Surface reactions such as oxidation or ionization are also important considerations in understanding how the surface differs from the bulk. Many metals and polymers have an oxidized layer. Surface oxidation increases the surface complexity of even the simplest polymers. This process, as will be shown, has many effects on the interaction of the material with cells and proteins. Furthermore, surface acidic and basic structures can exist in ionic or neutral form due to the material's environment in the human body.

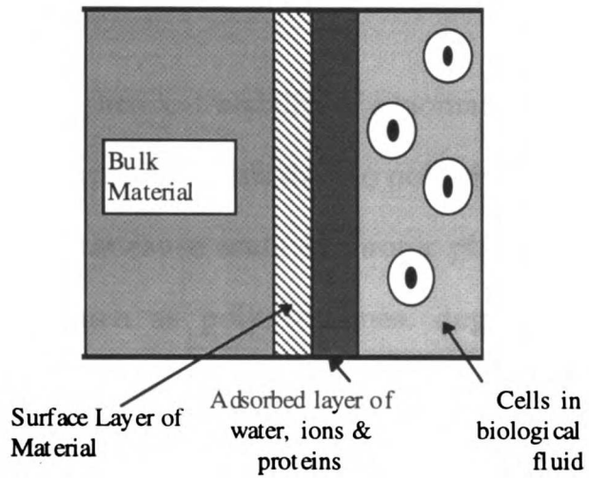
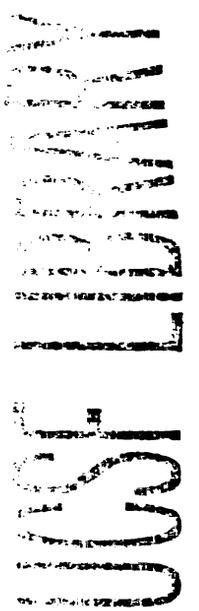


Figure 2.1 Biomaterial interface

Initial interaction of a material surface with a biomolecule can lead to permanent or temporary formation of: 1) weak van der Waals or hydrogen bonds or 2) stronger ionic or covalent bonds. Thus, interactions may be so strong as to reversibly or irreversibly denature proteins by breaking internal bonds of the protein in order to form bonds with the surface[Andrade, 1985]. These interactions may also lead to dissociation of small biomolecules such as H_2O and O_2, as well as hydrocarbons. Eventually larger structures such as cells will reach the biomaterial which is already covered with a surface coating or proteins. Since both the cell membrane with its coating of biomolecules and the material surface are dynamic, they can also exchange proteins, ions, and other molecules to form a complex and dynamic interface [Kasemo and Lausmaa, 1994]. Depending on the nature of the material surface and its protein coating, the cells will react differently. This difference is directly related to the biocompatibility of that material.

2.1.2 *Biostability*

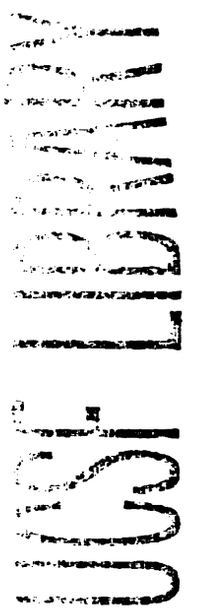
Biostability refers to the chemical stability of a biomaterial or medical implant in a physiologic environment. Materials that do not exhibit biostability often have degradation products that cause acute of chronic physiological damage. Most synthetic polymers, such as polyurethanes, degrade in the physiologic environment by mechanisms of nonenzymatic hydrolysis. Following surface hydrolysis, phagocytosis may take place if the particulate material is present from bulk degradation. This phagocytosis may generate free radicals and superoxide anions which further contribute to material degradation. Some synthetic polymers, such as those containing peptidic or glycosidic bonds, are prone to enzyme-catalyzed degradation. Silicon, on the other hand, has been found to be extremely inert in the biological milieu -- an important material property to consider in the design of a long term biocapsule implant.



There have been several studies on host reaction to particulate biomaterials. These host reactions are elicited by polymorphonuclear leukocytes, macrophages, mast cells, foreign body giant cells and fibroblasts. The interaction of the particulate surface with the particular cell dictates the extent of host reaction. An inflammatory reaction in response to particles is usually based on crystallinity and size. Although a majority of particulate materials induce inflammation, studies have shown that shistovite (SiO_2), anatase (titanium dioxide, TiO_2), and diamond are noninflammatory crystals [Mandel, 1976].

2.2 *Issues of Biocompatibility and Tissue Response*

When a biomaterial is implanted, a tissue response of inflammation and repair is observed. This response, similar in both animals and humans, is indicative of the biomaterial's toxicity. In simple terms, the characteristic tissue response to a non absorbable or nonbiodegradable material progresses from transient acute inflammation for less than a week to development of encapsulation by fibrous connective tissue after one month or so. Once formed, this fibrous capsule is usually stable and permanent, causing severe functional limitations for medical devices, especially those that are diffusion based.



The tissue response to implants is set in motion by cell death or necrosis upon surgical implantation and tissue disturbance. The onset of acute inflammation is triggered by degranulation of mast cells which leads to increased vascular permeability and increase blood flow at inflammatory sites. This facilitates the migration of polymorphonuclear leukocytes (neutrophils) and other inflammatory cells to the implant site.

Neutrophils are the most common cells present at inflammatory sites. They migrate to the implant area due to chemoattractant-secreting damaged cells. Upon removal of an implant or other cause of inflammation, neutrophils will disappear after 4 to 6 days. If marked neutrophil infiltration persists after one to two weeks, the implant may be toxic or infection may be present. The continual presence of neutrophils in tissues suggests a continued migration to the site, because of their less than 48 hour extravascular viability [Janoff, 1970].

The accumulation of leukocytes leads to abscess formation through leukocyte-mediated proteolytic digestion of local tissue.

Macrophages arrive to the implant site slower than neutrophils, but they are the promoters of tissue organization and repair. Activated macrophages are present at the surfaces of nearly all implanted biomaterials. However, the number of macrophages detected is proportional to the toxicity of that material. For smooth surfaced non-toxic biomaterials, there is a minimal macrophage population and by two weeks this population is largely replaced by fibroblasts and eventually a fibrous capsule. For implants with rough surface geometries or eliciting a toxic response, active macrophages will be present adjacent to the implant for several months.

Lymphocytes and plasma cells also play a key role in the inflammatory response. Lymphocytes, derived from bone marrow and lymph nodes, are present at sites of chronic inflammation and are characteristic of a prolonged immunological assault. Plasma cells, stemming from bone marrow derived lymphocytes, are the principal producers of immunoglobulins which are secreted at sites of chronic injury. Both plasma cells and lymphocytes predominate at sites that exhibit chronic infections or inflammation, especially in response to implants that have antigenic properties.

In addition to macrophages, the chronic inflammatory response can be characterized by the presence of fused monocytes and macrophages, known as giant cells. These cells are especially detectable in areas of granulatomous inflammation which most often leads to fibrous scarring. Materials that are toxic

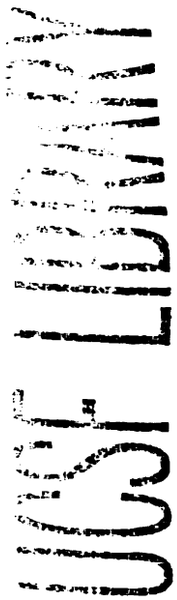
and non-biodegradable will attract permanent, large and irregular giant cells with as many as 200 nuclei that act as a barrier between the implant and the host tissue.

2.2.1 *Formation of Fibrotic Capsule*

The formation of a fibrotic tissue layer around cellular encapsulation devices has been a limiting factor in its therapeutic effectiveness and overall *in vivo* success. The granulatomous encapsulation tissue forms after the acute inflammatory response. This fibrotic tissue does not exhibit normal tissue architecture in that it generally lacks innervation and consists of large numbers of collagen-secreting fibroblasts, myofibroblasts, and chronic inflammatory cells. Such an architecture greatly limits diffusion of nutrients and other biomolecules to the encapsulated implant. Although a fibrotic response occurs to some extent with all implants, the thickness of the fibrotic layer depends on the size and surface properties of the implant [Salthouse and Matlaga, 1983] and increases with decreasing biocompatibility. Inert smooth surfaced implants such as teflon rods, stainless steel coupons, and silicon discs have been shown to be chemically nonreactive and thus, are surrounded by a thin layer of circumferentially oriented fibroblasts within 2 weeks of implantation [Woodward and Salthouse, 1986] with few, if any, inflammatory cells. In contrast, the response to surface-irregular implants that cause tissue aggravation consists of a fibrotic layer with many giant cells anchored to the implant.

Before any new material can be used in a medical device or implant, the biocompatibility of that material must be determined. The next section discusses

methods of biocompatibility testing that can be used in research. These methods often vary widely depending on material and investigator. Although in vitro testing is usually a good indicator of how a material will perform in vivo, ultimately the true test is implantation of the material itself and performance of desired function. Since it is difficult to predict the complex interactions of the body with that of a foreign material, direct implantation can provide information on both macroscopic and microscopic levels of the biocompatibility, biostability, and material toxicity.



2.3 *General Biocompatibility Testing Methods*

Testing the biocompatibility of materials is a rather inexact science. The definition of what constitutes a biocompatible material, although often debated, is usually regarded as a material that does not elicit any deleterious effects in a specific biologic application. Often times, the question is not whether there are adverse reactions to the material, but whether the material performs satisfactorily in the application under consideration.

The biological performance of a material can be divided into two aspects, that of the host response and the material response. Both of these aspects should be considered in relative terms rather than absolute because there is not always consensus over what the appropriate material/biologic response should look like.

Generally, studies on material compatibility follow the several basic steps. First, a material is chosen based on the engineering and material properties desired for the application intended. Then, it is determined in vitro, usually via

cell culture, whether the host response to that material is satisfactory and whether there are any changes in material properties. Finally, the response is verified during long term in vivo implant studies depending on application.

The use of cells in culture offers a relatively easy and inexpensive way to test the toxicity of biomaterials in vitro. Culture cells provide a homogeneous population of virtually identical genetic makeup in a constant and controllable ambient environment. The response of these cells to different environmental conditions and substrata can be measured by a variety of microscopic, histochemical, and biochemical techniques. Although cellular response does not necessarily correspond entirely to whole animal response, examination of isolated cells *in vitro* provides an essential starting point for biomaterials evaluation and is often times more sensitive to abnormal changes in cell physiology.

Mammalian cell culture techniques have been applied to study biomaterials for several decades. Exposure to biomaterials can be through direct contact with bulk materials, contact through agar or other gel substrates, or through incubation with material extracts or elutants. The cells in culture are then evaluated in terms of cell survival, cell reproduction, metabolic activity/functionality, or cell damage. The most common cell characterization procedures rely on morphological and colorometric measurements. In addition, protein analyses are often used to assay cell functionality.

Many cell culture models and cell types have been studied for use in testing device materials for biocompatibility [Hanks et al., 1996; Northrup, 1986].

Human fibroblasts or other mammalian cells are most commonly used. Cells or tissue samples that are isolated and cultured in vitro are termed primary culture. These cells can be grown on a glass or plastic dish with appropriate nutritional medium. Primary cultures can be obtained from digestion of tissue with proteolytic enzymes. These cells will then grow until confluence is achieved and further growth is obtained by subculturing in separate dishes with fresh media. Primary cultures can remain viable anywhere from a few days to several months, depending on the type of cell, tissue origin, and culture conditions. As the primary culture ages, cell growth is slowed until it completely terminates.

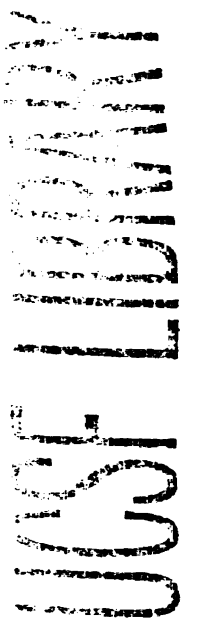
Continuous cell lines are obtained from primary cultures that have adapted to grow in vitro. These cell lines, unlike primary cell lines, can be cultured indefinitely under suitable conditions. Continuous cell lines are often cloned from a single cell to obtain cells of identical genetic origin.

Testing of the cells with the material can be done with two approaches: testing the material itself or an extract of the material. In the direct contact method, cells are cultured upon the test material and zones of inhibition or cell death are monitored. In the MEM Elution test, materials are extracted with MEM, a physiological cell culture medium, at 37°C for 24 hours, and this extract is used to feed a confluent layer of cells. Cells are examined for from 48 to 72 hours in terms of any morphologic or proliferative changes.

The complexity of the histochemical and histological response requires that potential implant materials also be evaluated for biocompatibility in vivo. In vivo implant tests can be divided into two types: nonfunctional and functional.

For the nonfunctional test, the implant is fabricated in a specific shape, and sits passively in several tissue sites. Such tests focus on the interactions of the material with both chemical and biologic components of the host tissue implant site. These tests are usually of six to twelve month durations, upon which the materials and host tissue is explanted and carefully examined.

The problem with in vivo testing is that there is little standardization of the test procedures. There are variations in surgical procedure, as well as variable factors such as species of test animal, age of animal, site of implantation, duration of experiment, and of course, material characteristics. For example, it has been observed that the implantation of almost any material into rodents results in neoplasia [Bischoff, 1973; Tang and Eaton, 1995] whereas, this same reaction may not be seen in larger animals and humans. Moreover, in determining the overall biocompatibility of an implant, several sites of implantation should be examined.

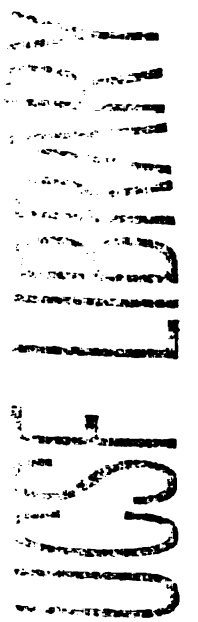


Evaluating the tissue reaction to an implant material is the primary means by which a material is deemed biocompatible. This can be done via a histological evaluation of types of cells present around implant site, the thickness of the fibrous capsules layer, or the extent of tissue ingrowth. The larger the reaction, the more intense the toxic response. As discussed earlier, almost any material will elicit some type of biologic response, so the end use of the material must be considered when evaluating its compatibility. Implantation of a period longer than 10% of an animal's lifetime is used for chronic implantation studies, in which both local and systemic effects can be evaluated. A three month implant

in mice or rats will produce the same local reactions regardless of total life expectancy [Wallin and Upman, 1996].

2.4 Silicon as a biomaterial

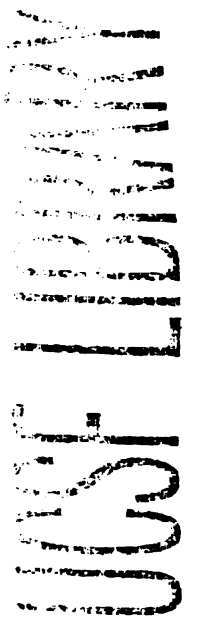
Prior to the onset of this research, there had been little investigation into the biocompatibility of silicon. As a semiconductor material, silicon has never been considered an optimal biomaterial, as compared to numerous other materials such as polymers, metals, and ceramics. Nonetheless, silicon has been shown in several studies to be extremely bioinert and non-toxic in cortical tissue, making it a potentially attractive biomaterial for use in biomedical microdevice fabrication. Studies on materials implanted into the cerebral cortex of animals showed that phosphorus-doped monocrystalline silicon was nonreactive in cortical tissue with the absence of any calcification, macrophages, meningeal plasma fibroblasts or giant cells in the connective tissue capsule [Stensaas and Stensaas, 1978].



Edell and coworkers (1992) showed that it was possible to design silicon structures which minimized tissue trauma when inserted into cortical tissue, which opened up the possibility of designing silicon microstructures for a variety of in vivo applications. Gliosis was observed at the array tips in long term studies which most probably indicated tissue movement relative to the tips. Only a single layer of tightly coupled glial cells surrounded the shafts of the arrays. It was determined that clear silicon dioxide coated structures inserted into the brain tissue of rabbits exhibited long term biocompatibility. Furthermore, healthy neurons were present in close proximity to the silicon array shafts. Edell

(1986) repeatedly demonstrated that, when stabilized, a biocompatible silicon interface with peripheral nerves could be maintained for several years. It became clear that one of the great advantages to using silicon microfabrication technology in neural recording and stimulation came from the ability to fabricate geometries on the same order of size as the nerve axons, with the flexibility of a incorporating a large number of electrode contacts.

Similar biocompatibility results were obtained by Schmidt et al. [1993] , who looked into the passive biocompatibility of uncoated and polyimide coated silicon electrode arrays in feline cortical tissue. They observed modest tissue reactions to the implants. Edema and hemorrhage were present around the short-term (24 hours) implants, but affected less than 6% of the total area of the tissue covered by the array. With chronic implants (six months), leukocytes were rarely present and macrophages were found around a third of the implants. Gliosis was found around all implants, but a fibrotic capsule was not always present and if so, never exceeded thickness of 9 microns. The amount of tissue reaction to the implants suggested that materials were non-toxic.



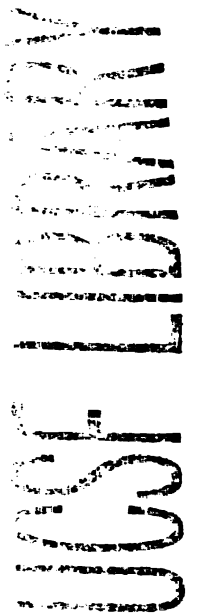
L.T. Canham (1995) recently began investigating silicon bioactivity with regards to in vivo bonding ability. In vitro studies on microporous silicon films in simulated body fluids showed that such films induced hydroxyapatite growth. He found that while bulk silicon is relatively bioinert, hydrated microporous silicon coatings were both biocompatible and "bioactive" with regard to hydroxyapatite nucleation. Results suggested that silicon could and should be

seriously considered for widespread in vivo investigations and applications as a biomaterial.

Indeed, in the 1970s and early 80s, elemental silicon was shown to be an essential trace nutrient [Schwartz and Milne, 1972; Carlisle, 1972]. Additionally, it was found that normal human blood serum and plasma contained silicon mostly as monosilicic acid ($Si(OH)_4$) at levels less than 1 mg Si/l [Carlisle, 1986]. Carlisle showed that even the complete dissolution of a 10 micron thick silicon atomic layer (2 x 2 x 0.5 mm slab) would only correspond to a total silicon ingestion of less than 1 mg (less than the reported bioavailability of silicon in a pint of beer).

Kawahara et al. (1965) was one of the first groups to develop highly detailed procedures for evaluating the biocompatibility of materials, including silicon, using tissue culture. They calculated several parameters to describe material biocompatibility, namely the cell outgrowth index, cell multiplication index, and cytotoxicity index. Much of Kawahara et al.'s work centered on the toxicity of pure metals and implant alloys. They found that the response of cells in culture to different materials corresponds strongly to the periodic law of elements. Specifically it was found that metals of group IIA and IIB inhibited cell multiplication completely and the cultured cells disintegrated. In contrast, elements of group IIIA, IVA, and IVB seemed to accelerate cell multiplication. These results made sense in terms of the electrochemical characteristic of the materials. For example, Groups IIA and IIB are easily ionizable materials and display rapid metallic corrosion which would induce chemical changes in culture

medium. Groups IIIA, IVA, and IVB, on the other hand, generally form a passivating oxide layer on their surfaces which prevent corrosion and maintains stability in culture conditions. Silicon was shown to have a cell outgrowth and cell multiplication index of 1.1 and 1.08 respectively, indicating cell stimulation, and a cytotoxicity index of -1 indicating non-toxicity.



3. Surface Interactions

3.1 Surface Characteristics for Improved Biocompatibility

There is now a great deal of interest in modifying surfaces of biomaterials to make them more biocompatible. Some of the surface modification techniques include coating, oxidation by low temperature plasma, and surfactant addition for anti-static and anti-dewing.

For medical devices that are inserted into body orifices, it is beneficial to have surfaces with decreased frictional resistances. This reduces damage to the body's mucous membrane. For example, hydrogel coatings have been tried to make the surface of catheters slippery and reduce frictional resistance between the catheter and mucous tissue [Ikada Y., 1994]. This can be accomplished by graft polymerization of non-ionic water soluble monomers to the device surface. The high water content of the grafted layer creates slipperiness.

Blood and tissue compatibility for biomaterials can be enhanced through several chemical and physical surface modification techniques [Nagaoka et al., 1995, Chesmel and Black, 1995]. One way is by covalently or non-covalently immobilizing bioinert hydrophilic polymer chains or heparin (anti-coagulant) molecules onto the material surface. Hydrophilic polymers that have been most commonly used include polyethylene oxide (PEO) and polyethylene glycol (PEG) [Llanos and Sefton, 1993; Mrksich and Whitesides, 1996; Jeon et al., 1991a]. These polymeric chains can be attached either through chemical coupling or physical adsorption.

Surface properties greatly influence protein adsorption and cellular reactions with biomaterials. Although a material alone may not elicit a strong cellular reaction, the extent to which it interfacially selects and concentrates specific proteins may determine their cellular reactivity. Although the adsorption of simple biomolecules at simple interfaces is well understood, there is still much uncertainty of the behavior of complex biomolecules at complex interfaces. There is a general lack of knowledge about molecular properties and processes at the biomaterial interface. It is, therefore, important to utilize all aspects of surface science in biomaterials research. This includes applying basic principles of surface-molecular interactions as well as detailed surface sensitive analytical techniques. Only then can we truly design biocompatible biomaterials for the human body.

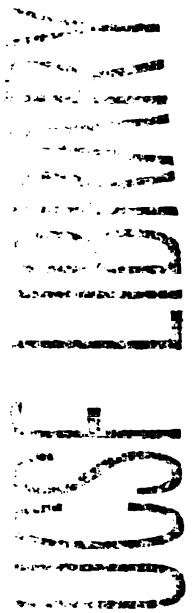
3.2 *Cellular Adhesion to Biomaterials*

There are three main types of cellular reactions with surfaces[Ratner BD, 1993]. Each of these processes is associated with the biocompatibility of the material. First, cells can strongly, but nonspecifically interact with a surface, leading to attachment and dedifferentiation of highly specialized cell types. The non-specific cell response can lead to the attachment of monocytes and macrophages, attraction of fibroblasts, and eventually fibrosis. Cells can weakly interact with a surface, specifically a nonadhesive surface. Here the foreign body response is minimized because there is no attachment. The cells are not activated to perform specific functions and therefore, the response is passive. Cells that strongly interact with a surface and specifically react with

the appropriate receptor sites, on the other hand, can perform highly specific functions.

3.2.1 *Surface Chemistry/ Functional Groups*

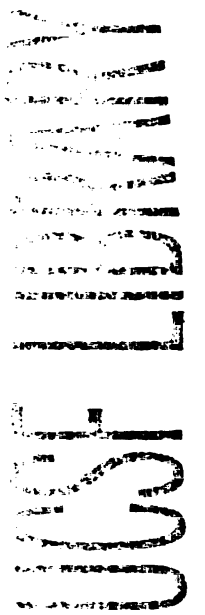
Because biomaterials encompass a diverse range of materials, it is important to examine the effects of different surface functional groups on cell adhesion and growth. For example, the charge of functional groups at the material surface has been found to be extremely important in cell adhesion and spreading upon that surface. The significance of substrate charge has been emphasized ever since the introduction of polystyrene culture dishes [Curtis et al., 1983]. Without surface treatments, polystyrene surfaces are unsuitable for cell attachment. Therefore, various treatments have been used by manufacturers to coat polystyrene surfaces for use in cell culture applications. It was found that the treatment of polystyrene with sulfuric acid introduces negatively charged hydroxyl groups and makes it suitable for cell attachment. In fact, almost all culture dishes are now chemically modified in some manner before use.



Since studies have shown that cells adhere to both positive and negative surfaces, cell attachment to a substrate is not only a simple electrostatic attraction between a negatively charged cell membrane and a positively charged surface, but some additional factors as well. It seems that cell attachment is also dependent upon the presence of a critical number of charges [Rappaport et al., 1960]. The fact that cells will adhere to either positively or negatively charged surfaces has to do with charge density [Maroudas NG, 1975].

Indeed, studies have confirmed that charge density, rather than sign, at the solid surface is more important in cellular adhesion.

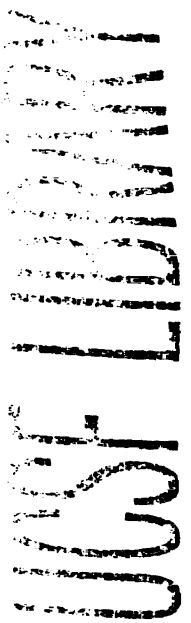
In addition, cells have been found to be more adherent to surfaces with hydrophilic moieties because these groups increase the wettability of the surface. A hydrophilic solid surface is one on which water will spread and be absorbed into the bulk of the material. This is in contrast to a hydrophobic solid surface on which water and aqueous fluids will not spontaneously spread but will form separated drops having a non-zero contact angle, i.e. nonwettable. Kawahara et al. (1968) found that the contact angle between cells and the substrate decreased proportionately with an increase in hydrophilic radicals in various polymers. Although hydrophobic surfaces strongly interact with proteins, cells are not as adherent to these surfaces primarily because no hydrophobic bonding occurs to drive the cell-material interaction.



For surfaces with neutral functional groups such as amide and hydroxyl groups, the hydroxylated surface tends to show better cell adherence probably due to specific hydrogen bonding between surface hydroxyl groups and the polar groups of the cell surface [Lee et al., 1994]. Functional groups, such as amine which are positively charged in pH 7.3-7.4, seem to show the best cell adhesion. It has been recognized that a large area of the cell membrane and serum protein surface is negatively charged. This results in the electrostatic interaction of the cell surface with the positively charged material surface[Lee et al., 1994]. However, negatively charged functional groups such as carboxylic groups have shown poor cell adhesion. So, although wettability is important

in cell adhesion, surface chemistry and charge character are also very important factors.

In addition to surface charge, it is found that the overall viability of cells is affected by other electrochemical characteristics of a material surface. For example, metals of groups IIA and IIB seem to inhibit cell multiplication and degenerate and disintegrate cells [Hench and Ethridge, 1982]. On the other hand, metals of groups IIIA, IVA, and IVB tend to accelerate cell reproduction. This can be explained by noticing that metals of groups IIA and IIB are ionized easily and quickly corroded. These metals cause an electrical double layer with a negative charge. The nobler metals do not ionize easily. The chemical change of corrosion affects the pH of the biologic environment, thus having harmful affects on the cells. Also, electrical currents may affect cell migration and metallic ions may affect cellular metabolism.



Corrosion resistance of implant surfaces is achieved by the formation of oxides or compact solid films of hydroxides on the surface. These passivation films maintain equilibrium with oxygen in the local environment. Moreover, these passivating layers usually have no adverse affect on cells. In fact, a decreasing contact angle and more cellular adhesion has been observed with increasing oxide layer thickness[Sharma and Paul, 1992]. However, for some materials the presence of chloride ions can disrupt the passivation of the surface. These anions adsorb onto the material and can break down passive films. Therefore, one must be careful when using corrosive materials in a physiologic environment.

Another factor that influences material properties in vivo is backbone and side chain molecular motions of the surface. Studies indicate that the initial interaction of cellular elements onto the surface of materials is mediated in part by the relative degrees of surface molecular mobility associated with side chains. In most cases, when molecular motions are restricted, less cellular response is observed. For example, a decrease in thrombogenicity has been observed by irradiating polymeric materials (irradiation creates partial restriction of side chain motion) [Reichert et al., 1982]. This occurs independent of morphological order/disorder, crystallinity, and associated water.

Recently, the importance of microtexture and microporosity in materials has also been realized. It has been shown that porous materials with small pore sizes and without long, oriented attachment surfaces for cells, leads to good healing responses characterized by the absence of a fibrous capsule [Guenard, Valentini, and Aebischer, 1991]. The cells in contact with such biomaterial surfaces remain rounded as if they were not seeing any surface at all. In terms of biocompatibility, it means that the surface of the material interacts with no adverse effect on the biological environment.

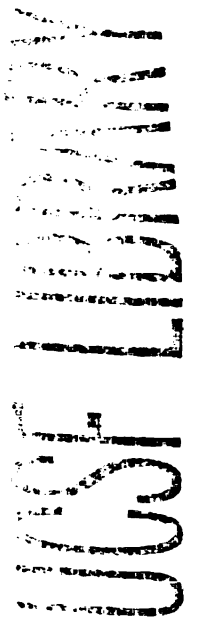
3.3 *Protein Adsorption onto Biomaterials*

As discussed previously, a biomedical implant induces two main types of reactions: foreign body response occurring around soft tissue implants and thrombosis on surfaces in contact with blood. Although both reactions involve the interaction of cells with the surface, it is the initial adsorption of proteins to the interface which mediates cellular response. Therefore, it is first important

to understand the interaction between surfaces and proteins in order to study subsequent cellular events in the biocompatibility process.

3.3.1 *General properties of the protein-biomaterial interface*

Proteins are intrinsically surface active and tend to concentrate at the surface of the implanted material [Kasemo and Lausmaa, 1994]. This is partly due to their polymeric structure and their amphipathic nature. Because of the large size of a protein molecule, it can have multiple contact points with the interface and this increases the tendency of the molecule to remain at the surface. Proteins can have polar, charged, and nonpolar side groups which enable them to bind with many different surfaces. Each protein has its individual surface chemistry produced by the outer shell of amino acids and carbohydrate which interface with the liquid medium. Although protein molecules can collide with material surfaces in many different orientations, one specific orientation will result in the most stable adsorption. In a native protein, nonpolar groups tend to be internalized. However, when in contact with a surface, the protein can alter its structure to achieve maximum contact with the surface.



A schematic view of a four-sided protein is illustrated in Fig. 3.1. The protein is shown with one face hydrophobic, one negatively charged, one positively charged, and one face of neutral hydrophilic nature. They are shown interacting with surfaces of complementary character. The neutral material surface shows little adsorption while the hydrophobic surface has strong adsorption in the orientation shown. This is due to strong hydrophobic-

hydrophobic interactions in an aqueous biological environment. Charged surfaces, on the other hand, have variable adsorption depending on the electrostatic interaction of the protein and surface. This interaction can be a function of such parameters as the ionic strength, pH of the solution, charge density, and charge location. This will be described in detail later.

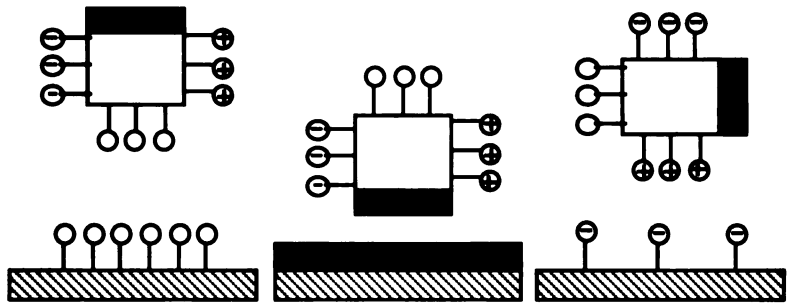


Figure 3.1 Schematic view of four sided protein interacting with various surfaces

In general, the local concentrations of proteins at interfaces greatly exceeds concentrations in the bulk phase. This concentration of proteins at the interface facilitates the ability of cells to react with proteins since interaction with closely spaced protein molecules by the cell can enhance binding.

3.3.2 *Composition of the adsorbed surface layer*

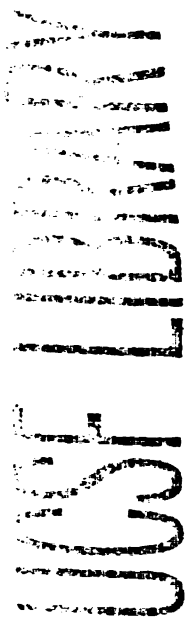
Site density and reactivity are the main properties of the surface that influence the composition of the adsorbed protein layer on a biomaterial. The differing concentrations of various proteins in the adsorbed layer of

biomaterials may explain why some surfaces elicit more intense cellular reactions than others. This poses the question of what differences in surfaces elicit differences in cellular response? Studies have demonstrated that there are several differences. For example, since a cellular response requires the close spacing of similar molecules on the interface to bind and trigger the cell, the site density of proteins on the surface seems to play a key role. Depending on the material, it has been shown that albumin adsorption can range from .2 ug/cm^2 on hydrophobic Teflon (Poly tetra fluoroethylene) to 0.06 ug/cm^2 on Biomer (a polyurethane) [Horbett, 1981].

Adsorption studies of protein solutions provide the most information as to the composition of the adsorbed protein layer. The layer is quite complex with as many as ten different proteins in the adsorbed layer. The composition reflects differences in surface activity and bulk concentration of the proteins. However, there is not just one dominant protein on the surface. Although fibrinogen and hemoglobin are in relatively small concentrations in solution, they are present in the adsorbed layer on a biomaterial due to their high surface activities. On the other hand, albumin is also present because of its high concentration, despite its low surface activity[Weathersby et al., 1976]. Looking at several kinetic studies of the initial adsorbed protein layer, it seems that there is rapid rearrangement of the adsorbed layer, depending on the nature of the surface. On each type of material, there exists a unique organization of proteins.

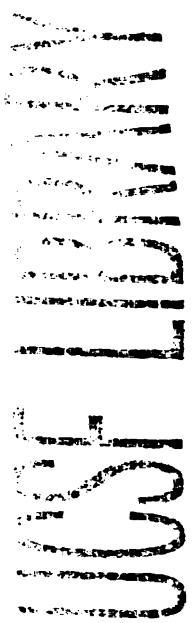
3.3.3 *Structure of surface-adsorbed proteins*

Proteins tend to become denatured or unfolded at material interfaces. This leads to the idea that the extent to which a protein is denatured by the surface leads to differences in cellular response by that surface. There is still uncertainty as to the degree of denaturation of adsorbed proteins. In a study with γ-globulin adsorption onto polystyrene, it was found that very high molar heats of adsorption occurred at lower degrees of adsorption. This indicated that there were substantial conformational changes. But, the heat of adsorption decreased greatly with high bulk concentrations and high degrees of adsorption [Horbett, 1982; Szleifer, 1997].



Proteins are adsorbed on the surface of materials by attachment of various segments along the chain. Attached segments are either single or in series with loops extending out of the surface into the solution. The size and distribution of the loops depends on the protein-surface interaction energy. For adsorbed proteins, numerous internal constraints due to disulfide linkages, hydrophobic, and electrostatic interactions limit the set of conformations upon the surface. The extension of the adsorbed protein molecules increases with decreasing surface free energy. It was found that the adsorbed protein thickness increased from 32 to 59 nm over the following range of materials: platinum, chromium, quartz, carbon, and polyethylene[Norde, 1992]. This agrees with the theory that proteins have a tighter conformation on high-energy surfaces and a more open conformation on low-energy surfaces.

Although proteins can change their structure upon adsorption, they usually remain relatively compact in the adsorbed state[Barbucci and Magnani, 1994; Norde, 1986]. It is shown that if a net amount of electrical charge is present in the low dielectric contact region between the sorbent surface and the adsorbed protein, this leads to very high electrostatic potentials which are unfavorable. This accumulation of net charge in the inner region of the adsorbed layer is avoided by incorporating small ions from solution[Norde and Lyklema, 1978]. As the uptake of charge neutralizes opposite charges, it is electrostatically favorable. However, the change in the chemical environment of transferring ions from an aqueous into a nonaqueous layer is unfavorable and opposes the overall protein adsorption process. Thus, maximum affinity for protein adsorption is observed under conditions where the charge of the protein molecule matches the charge on the sorbent surface[Norde, 1992].



3.3.4 *Thermodynamics of protein adsorption onto biomaterial surfaces*

Although protein-interface behavior has been studied extensively over the years, it still appears to be an area of much controversy. There are, however, some general characteristics of the interaction. The interaction between proteins and the material surface is primarily determined by 1) changes in the hydration of the sorbent surface and the protein molecule; 2) Coulombic interactions between the protein and the sorbent, which lead to the redistribution of charged groups; and 3) structural rearrangements of the protein molecules themselves upon surface adsorption.

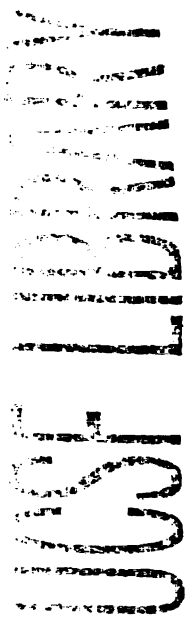
Protein adsorption can be seen in standard thermodynamic terms. The process can occur only if the Gibbs free energy of the system decreases,

$$\Delta G_{ads}=\Delta H_{ads}-T\Delta S_{ads}<0$$

We can look at the process of protein adsorption from solution onto a solid substrate as the change in free energy

$$\Delta G_{ads}=g_{ps}-g_{pl}-g_{sl} = -W_{ads}$$

where g_{ps}, g_{pl}, and g_{sl} are the protein-solid, protein-liquid, and solid-liquid interfacial tensions, respectively. The change in free energy of the system is equal to the protein-solid work of adsorption, Wads. So, when $\Delta G_{ads} < 0$ and $W_{ads} > 0$, adsorption is favored. In biological systems, which are essentially in aqueous media, protein(polymer)-water interactions are important to consider since protein affinity for a surface is related to these interactions.



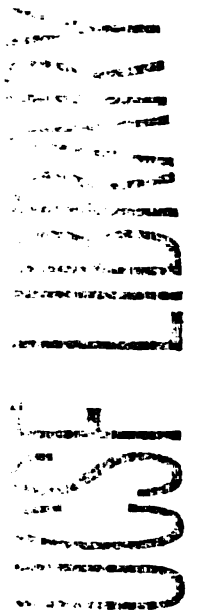
In much of the literature, protein adsorption onto biomaterial surfaces is treated with simple models, such as Langmuir isotherms. However, this implies that there is localized reversible adsorption without lateral interactions which is usually not the case with proteins. As a rule, protein sorption is not reversible with respect to concentration changes. Hysteresis between adsorption and desorption onto a biomaterial surface is indeed observed. The desorbing protein molecule differs from the adsorbing one because the Gibbs free energy of the desorbing molecule is more positive that that of the protein before adsorption. This means that there is a creation of entropy upon protein

adsorption[Norde, 1992]. This is in agreement with the observation that desorbed proteins have decreased secondary structure.

Furthermore, it has been shown that proteins change conformation upon adsorption. Norde and Lyklema (1979) analyzed the adsorption of albumin onto polystyrene and found that the important contributions to DG were dehydration of charged groups and rearrangement in the protein structure and hydration. They verified that the Gibbs free energy of hydration becomes more negative with increasing hydrophobicity. So, for a hydrophobic polystyrene surface at 25°C, ΔG_{dehyd} = -16.8 mJ/m^2. This comes from a large entropy contribution $T\Delta S_{dehyd}$=14.4 mJ/ m^2 and small enthalpy effect ΔH_{dehyd} = -1.4 mJ/m^2. For a 1.5 mg protein with a molecular weight of 50 kD adsorbed on a square meter of surface, ΔG_{dehyd} would be -226RT per mole of protein . This large negative value explains why all proteins adsorb strongly at hydrophobic surfaces, even under unfavorable electrostatic conditions.

The overall change in free energy from the redistribution of charged groups also contains terms for electric and chemical contributions from ion incorporation, ΔG_{el} and ΔG_{chem} [Norde, 1992]. However, because ions are often incorporated in the protein-surface contact region, as described earlier, ΔG_{el} does not have large values. The value of ΔG_{el} depends on the composition and the dielectric constants of the electrical double layer before and after adsorption. In addition, for most low molecular weight ions, $\Delta G_{chem} > 0$. This is

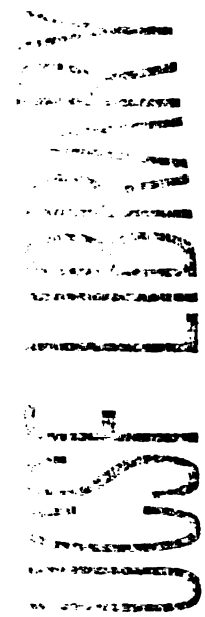
a result of negative values for both ΔS_{chem} and ΔH_{chem}, or a large negative value for ΔH_{chem}. This would seem to oppose the overall protein adsorption process. So, a way that protein-surface systems can avoid high electrostatic potentials, is to have unfolding of the adsorbed protein into loosely coiled conformations that are penetrable by small ions and water molecules. This creates a highly hydrated adsorbed layer where the dielectric constant is close to that of the bulk solution. However, the chemical effect of ion incorporation is still less unfavorable than exposure of hydrophobic parts of the protein that would occur upon unfolding.



In most cases, protein-surface adsorption isotherms develop plateaus. These plateaus reach a maximum at the isoelectric point of the protein-sorbent complex [Elgersma et al., 1991]. It seems that proteins with a stable structure have an adsorption onto a surface that is governed by electrostatic interaction and changes in the hydration of the surface and protein. In contrast, those proteins with low structural stability have the additional driving force for adsorption due to changes in structure. Therefore, even under the condition of electrostatic repulsion , hydrophilic surfaces may adsorb unstable proteins. In a mixture of four proteins, it was observed that the least stable protein adsorbed preferentially on all surfaces [Arai and Norde, 1990].

So, overall the protein-material interface depends on many aspects including adsorbed amount, orientation and structure of the adsorbed layer, and individual functionality of the biomolecules themselves. By being able to characterize the type of protein interaction that will occur at a material

interface, one can gain an understanding of not only how the cells of the body will respond to the material but also how the material will react to the body.



4. MEMS : Micro-electro-mechanical systems

The miniaturization of technology used to be only a futuristic dream seen in science fiction movies and animation. Sub-micron submarines cruising the body and attacking foreign invaders, high precision sensors monitoring one's every movement, bionic arms and limbs, and virtual eyesight through artificial eyes -- ideas many considered fanciful have now found basis in present day technology, much of which is MEMS based.

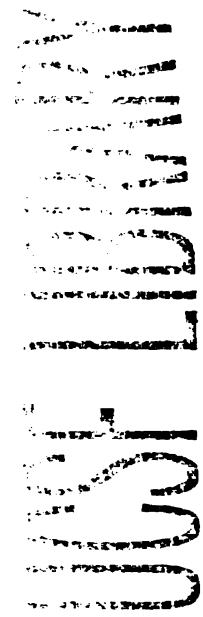
4.1 The Micromachining Revolution.

Technological advances in the fabrication of micro-electro-mechanical systems have significantly contributed to the emergence and rapid development of a new discipline aimed at developing microfabricated devices for biomedical applications. This technology, now commonly referred to as BioMEMS, uses protocols and fabrication techniques that were originally developed for integrated circuits (IC) industry to make intricate miniaturized mechanical structures on the surfaces of silicon wafers. These miniature devices have ranged from electro-mechanical systems such micromotors [Howe and Muller, 1986; Mehregany, 1990], or microtweezers [Chen and MacDonald, 1991] to more structural systems such as micromachined filters [Ferrari, 1995].

In general, a principal goal of MEMS technology is the miniaturization of mechanical structures that perform the same or improved function as their larger counterparts, but with a significant reduction in the size and mass of the device. These miniature devices can be integrated with circuitry and signaling

capabilities in order to perform specific analytical or diagnostic functions. It is now possible to successfully integrate more than a million transistors on a single silicon chip, allowing sophisticated control at low cost [Wise and Najafi, 1991]. Due to these technological developments in the field of MEMS, the last two decades have witnessed the explosion of a related technology, that of sensors and actuators. These devices are able to interface circuitry with the outside world in order to create useful tools for applications in everything from automotive systems to industrial automation.

In the 1960s and 70s, considerable progress was made in developing micromachining techniques for sensors and detectors, which facilitated the shift of technology from the laboratory to widespread production. With the development of surface micromachining in the next decade, it was possible to merge circuits with microsensors and actuators without previous signal-to-noise and packaging problems. Such devices as flow meters, accelerometers, and optical detectors emerged.



4.2 *BioMEMS*

More recently, the technology of MEMS has been channeled towards applications in biology and medicine. The race to find effective diagnostic and therapeutic tools is underway, as scientific and engineering disciplines uncover and elucidate more about the human pathologic condition than ever before. Moreover, the human body seems appropriate as a target of MEMS technology since most structures in the body are in the micron to millimeter size range, the same size range as most MEMS devices. In no other technology is it possible to

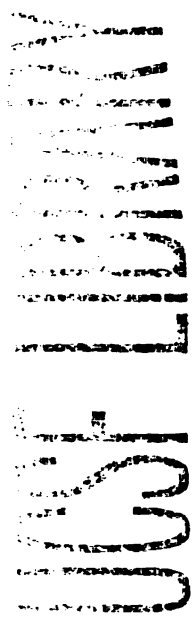
fabricate device features spanning three or more orders of magnitude, with both precision and accuracy, in the same fabrication process. Moreover, the miniaturization of both diagnostic and therapeutic devices facilitates their use in the body.

Probably the most common biological application of MEMS is the miniaturization of biosensors for portable analysis units. Biosensors are a combination of a transducer and a selective biorecognition system, which is typically an enzyme, antibody, or animal cell. Biosensors can be *potentiometric* in which they sense changes in concentrations of protons, ions, or other indicators, or *amplifying* in which they augment the concentration of analytes for detection.

Microfabricated sensing devices has allowed the creation of miniature sensors that can be used within the body without greatly disturbing the natural processes [Wilkins, 1995]. Ko *et al.* (1979) developed one of the first microfabricated piezoelectric sensors for long-term monitoring of cardiovascular and intercranial pressure and short-term monitoring of respiratory and gastrointestinal pressure. Although there were several problems due to its non-biocompatible packaging, it initiated further investigation into the use of microfabricated devices for biomedical applications. Subsequently, MEMS were investigated for use in the recording and stimulation of neural signals through micro-transducers. Different designs of neural actuators, including textured plate electrodes [Ziaie, 1997], arrays of needle electrodes [Normann, 1991], and "sieve" electrodes [Akin, 1991] were

introduced. All designs delivered signals to or from neurons interfaced through silicon microsensors.

In addition to sensors, several other types of biomedical devices are rapidly being developed such as instruments for central nervous system (CNS) stimulation and recording [Chen and Wise, 1997; Edell et al., 1992], neural regeneration technology [Akin et al., 1994; Kovacs et al, 1987], and on-chip electrophoretic diagnostics [Wooley et al, 1997]. Recently, silicon microstructures have been used for cell guidance[Mrksich and Whitesides, 1996; St. John et al. 1997] and the handling, manipulation, and monitoring of single cells in culture[Feong et al., 1993; Jimbo et al., 1993]. The development of the microfabricated biocapsule, as presented here, is a step towards creating BioMEMS for *therapeutic*, rather than only *diagnostic* applications.

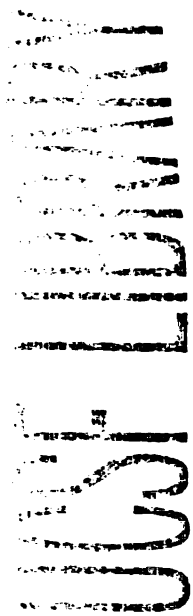


4.3 *Silicon Micromachining -- An Overview*

In order to design a microfabricated biomedical device, it is essential to understand the potential capabilities and limitations of the technology. In the past three decades the element silicon has become the material of choice in the microelectronics industry, primarily due to its electrical and machining properties. The recent development of a set of techniques collectively called micromachining allows the fabrication of silicon into micro-mechanical devices in the same size range as micro-electrical ones. The lower limit on device size arises from the limitations of the microfabrication facilities. Currently, in industry, the lower limit is about 0.15 μm, whereas in universities such as UC

Berkeley, the minimum linewidth associated with photolithography is 0.6 μm [Microlab Manual, 1995]. Mechanical systems such as valves and nozzles as well as other three dimensional shapes can be chemically etched in wafers of single crystal silicon.

Micromachining makes use of and extends fabrication techniques commonly employed in the integrated circuits industry. Fabrication relies on the depositing and selective etching of thin film layers. There are two main types of materials used in microfabrication of MEMS: structural materials and sacrificial materials, although the same material may fall into both categories in different applications. The difference between the classifications comes from the utilization of the material in the final device. Structural materials, as opposed to sacrificial materials which are etched away, are those that comprise the final structure of the devices once processing is complete. By combining various structural and sacrificial layers and elements, complex microdevices can be constructed. However, the current limitations of microfabrication technology only allow for a restricted choice in materials (several insulators, semiconductors and metals). Since silicon and silicon dioxide currently represent the most common materials for MEMS fabrication, these materials became the starting material from which to fabricate the biocapsule.

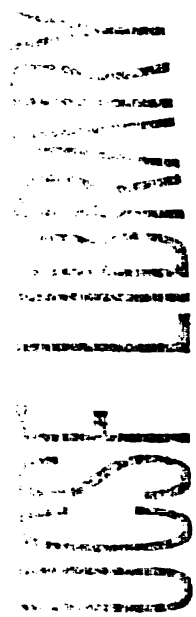


4.3.1 *Material Properties of Silicon*

Silicon , a semiconductor material, is readily oxidized, a property that is extensively utilized both in micromachining and microfabrication. The silicon dioxide surface layer that readily forms is chemically inert and electrically

insulating. Therefore, these layers are commonly used during the fabrication of microelectronics.

A semiconductor is a solid in which the highest occupied energy band at T=0 degrees Kelvin is completely filled and the next band (called the conducting band) is completely empty of electrons. The lowest energy level in the conduction band is separated from the highest energy level in the valence band by only a small gap. This energy or "band" gap is a direct measure of the amount of energy an electron needs to leave the bonds. The band gap is different for different materials.



Electrons in the conduction band can accelerate because empty, higher energy levels are available to accommodate them. If a hole exists at the top of the valence band, an electron in a lower level can be given energy to fill it and the accelerating electron carries a current. Semiconducting behavior is determined by the number of electrons in the conduction band and the number of holes in the valence band. The electrons and holes may be created by excitation across the band gap or by the introduction of suitable impurity elements into the lattice.

Semiconductors show much larger thermoelectric effects than metals and are used in a wide range of devices such as temperature sensors, thermopiles, and thermoelectric refrigerators. The energy for the excitation of charge carriers in semiconductors may also be supplied by electromagnetic radiation, particularly in the infra-red and visible regions. Hence, various photoelectric devices are also possible with semiconductors.

Some other semiconducting elements include boron, carbon, germanium, tin, phosphorus, and arsenic. However, Silicon (Si) and germanium (Ge) are the most frequently used elemental semiconductors. Silicon is the most widespread semiconductor used for digital electronics because Si is cheap and abundant, it is structurally robust, and from an environmental point of view, it is relatively harmless.

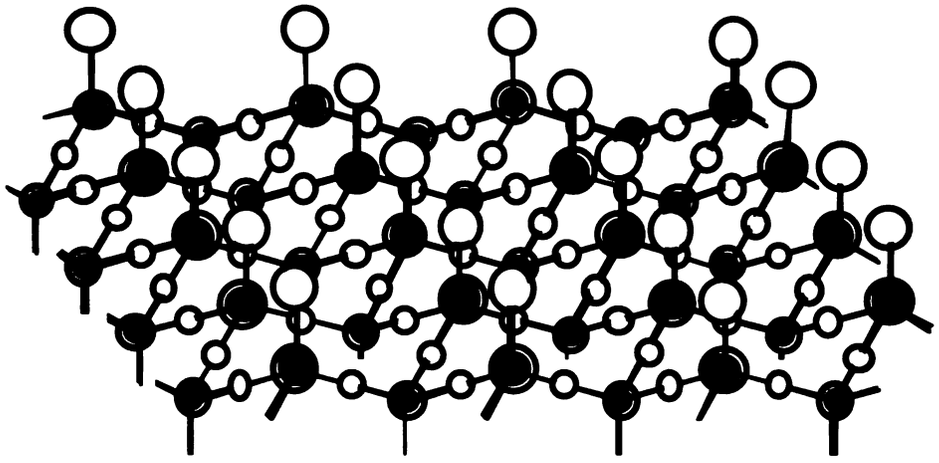
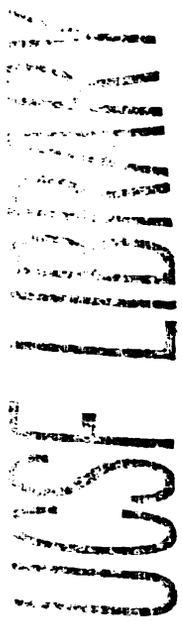


Figure 4.3.1 Schematic of Atomic Structure of Silicon

Silicon has a diamond cubic unit cell, in which each silicon atom is tetrahedrally coordinated with four neighboring atoms with which it is bonded. One can think of the silicon crystal having an interlocking face centered cubic structure. The interatomic bonds of silicon are covalent and slightly weaker than those of diamond. The covalent bonding between silicon atoms leads to a stable internal configuration. The surface atoms, on the other

hand, have unattached "dangling" bonds when in vacuum, and rapidly attract and bond with oxygen molecules in air or water. Mechanically, single crystal silicon is brittle, but it is also harder than most metals. It is has been found to be extremely resistant to mechanical stress and in tension and compression, it has a higher elastic limit than steel. Silicon, as a single crystal, is also strong under repeated cycles of tension and compression.

Connally and Brown [1992] studied the mechanical properties of silicon-based micromechanical devices, realizing that macroscale properties do not necessarily translate to the microscale. It is at the small scale of microdevices where surface coatings, microstructural features, and interfacial properties become significant relative to the device dimensions, and continuum assumptions do not always apply. They found that certain environmental conditions such as those found within the human body can accelerate crack growth, once initiated.



4.4 Microfabrication and Micromachining Techniques

Microfabrication for biomechanical devices utilizes much of the same technology that is the basis for the microelectronics industry. The basic processes include lithography, thermal oxide growth, diffusion, deposition, and etching [Jaegar, 1990]. Additional issues often present in BioMEMS fabrication include biochemical surface modification, bonding and sealing, as well as assembly. Maintaining and preserving device biocompatibility is crucial for all processing steps.

The prefix in *microfabrication* refers to the typical limit of resolution that can be achieved, which is usually on the order of microns. However, with some specialized techniques and creativity, typical microfabrication protocols can yield nano-sized features. As will be discussed, the microfabricated biocapsule utilizes several novel microfabrication techniques to create nano-sized pores. All microfabricated structures are created on or within a silicon wafer (usually in plane).

For simplicity, microfabrication processing technology can be thought of in terms of four basic operations: addition, subtraction, multiplication, and division [Ferrari, 1996]. Addition refers to thermal growth or deposition of various layers on the wafer or current surface through a number of processes such as evaporation, sputtering, or chemical vapor deposition. Subtraction, on the other hand, is the removal of carefully defined portions of the deposited layer, usually by use of photomasks and photolithography. Removal can be done by dry plasma etching or wet chemical etching which will be described in further detail later. The replication of single dies across to wafer to produce large scale exact reproductions is the multiplication operation. And finally, division involves wafer separation into individual dice, and their subsequent assembly and packaging. Although this is a simplification of microfabrication, it serves as a useful way to understand microfabrication in relation to the development of BioMEMS technology.

4.4.1 Processing Steps

The first step in microfabrication usually involves growing a thin oxide (SiO_2) layer on the surface of the wafer. There are several reasons why a SiO_2 layer is important. This layer can be used as a sacrificial layer, structural layer, diffusion barrier, insulator, or as the basis for biochemical modifications. The layer is usually achieved by heating the wafer surface to between 800 and 1200 degrees Celsius in steam. Dry oxygen can also be used, although it results in much slower oxide growth. The chemical reaction occurring in the two different conditions is:

Water vapor: $Si + 2H_2O \rightarrow SiO_2 + 2H_2$

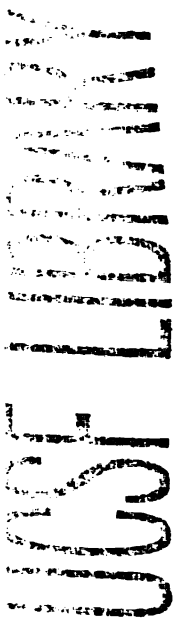
Pure oxygen: $Si + O_2 \rightarrow SiO_2$

It is important to note that a thin (usually a few nanometers) native oxide layer will develop naturally when a silicon surface is exposed to air or a hydrated environment. In general, the thermal oxidation growth rate is controlled by pressure. The substrate, its crystal orientation, and doping also influence the oxidation rate.

Besides the oxide layer, many other layers or films can be created from a variety of techniques. There are four broad categories of film deposition methods: evaporation, sputtering, chemical vapor deposition, and epitaxy. Evaporation is performed in vacuum and involves heating a metal to vaporization. Subsequent solidification results in a film forming on a deposition substrate. The main evaporation methods are: filament heating,

high-energy beam focusing (i.e. E-beams or lasers), and flash (contact) evaporation.

Sputtering involves bombarding a target with high-energy ions, typically Ar^+. A wide range of materials can be deposition through this method, including conductors such as Al, Ti, and W. This technique has the advantages of being composition preserving, providing better surface coverage, and not producing radiation damage. However, heating of the substrate and incorporation of argon can be disadvantageous in some applications. Alternatively, single crystals can be grown by epitaxy through vapor phase, liquid phase, or molecular beam epitaxial methods. Single crystals can be grown monolayer by monolayer on the substrate. Although there is great control with this technique, the throughput is low and it is quite expensive.



Chemical vapor deposition (CVD) can be performed at both low pressure (LPCVD) or atmospheric pressure. It involves flowing reactant gases on substrates, where they then create thin films by thermal decomposition and/or chemical reactions. Materials that can be CVD deposited are oxides and metals, or any material with the proper precursor gases. In addition, polysilicon can be deposited by LPCVD through thermal decomposition of silane at temperatures of about 600 degrees Celsius. The chemical reaction is : SiH_4 Si + $2H_2$. The temperature and pressure of deposition greatly influence the polysilicon microstructure (grain, size, and texture), the residual stress

portrait, as well as the growth rate. Typical growth rates are from 10 to 20 nanometers per minute.

Subtraction or removal of a portion of the deposited layers or wafer itself are performed by chemical or plasma etching. During the etching process, parts of the wafer are protected and not removed. To define these protected regions, the technique of photolithography is used. The limit of the resolution that be accomplished with lithography is determined by the wavelength of the light used and the optics used to focus the light after it passes through the mask. For the near UV light used with the GCA wafer stepper in the Berkeley Microfabrication Lab, the theoretical feature minimum size is 0.6 μm [Microlab manual, 1995]. The lithographic technique ultimately results in copies of a master pattern being transferred onto the surface of the silicon wafer. After a barrier layer is deposited (i.e., SiO_2, Si_3N_4, metal), a thin layer of an organic polymer is deposited. The surface must be clean and dry to ensure good adhesion of the polymer. This organic polymer, termed photoresist, is sensitive to ultraviolet radiation. It is usually in liquid form and applied to a wafer spun at high speed (1000 to 5000 rpm for 30 to 60 seconds) to produce a thin uniform layer (2.5 to 0.5 microns). A drying step called soft baking is used to improve polymer adhesion and remove solvent from the photoresist. The photoresist is then ready for the photomask step.

A photomask is placed in close proximity to the photoresist. The photomask is usually a transparent glass plate with an opaque metal pattern. Each mask must be carefully aligned to the pattern on the wafer. When the

wafer is exposed to UV radiation, a chemical reaction occurs in the exposed areas of photoresist (i.e. the areas not covered by the opaque metal of the photomask). If positive photoresist is used, the chemical reaction weakens the polymer. Negative photoresist, on the other hand, strengthens the polymer. After exposure to UV radiation, the wafer is rinsed in developer. This either removes the exposed or unexposed areas of photoresist, leaving a pattern of bare silicon and photoresist coated oxide on the silicon wafer.

Chemical etching in liquid or gaseous form is used to remove any barrier material not protected by the hardened photoresist. The choice of which chemicals to use depends on the material to be etched, as well as the shape desired. A high degree of selectivity is required so that the etchant will remove the unprotected layer more rapidly than the photoresist-protected layer. Essentially, the photoresist acts as a protective shield for the area it covers. When the unprotected regions have been stripped away, the protective photoresist can be removed by hot sulfuric acid, which attacks the photoresist, but not the oxide or silicon underneath. The result is an oxide pattern that duplicates the photoresist pattern and is either a positive or negative copy of the photomask pattern.

There are two major etching processes: wet chemical etching and dry etching. Wet chemical etching involves the immersion of a wafer in a selectively etching solution. For example, a buffered solution of hydrofluoric acid can be used to attack the oxide but not the photoresist or the underlying single crystal silicon . The etch rate is strongly dependent on temperature and

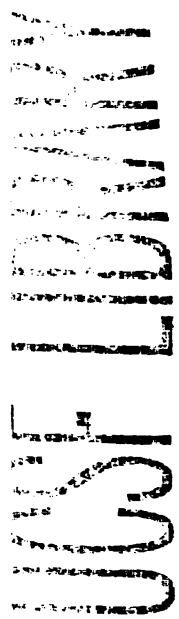
impurity (doping) concentration. For example, BHF is highly specific to silicon oxides, having an etch rate ratio of 1000:9 for silicon oxides to silicon [Microlab, 1995]. Dry oxygen-grown oxide etches slower than water vapor-grown oxide; phosphorus impurities accelerate the etching while boron doping slows it down.

Dry etching processes are used to obtain highly anisotropic etching profiles, by avoiding the undercutting problems characteristic of wet processes. Dry processes use only small amounts of reactive gases, whereas wet etching techniques require relatively large amounts of liquid chemicals. One type of dry etching is plasma etching. In this process, wafers are immersed in a gaseous plasma created by radio-frequency (RF) excitation in a vacuum system. The plasma usually contains fluorine or chlorine atoms which etch silicon dioxide. Another process, sputter etching, uses energetic noble gas ions such as Ar^+ to bombard the wafer surface. Etching takes place by knocking atoms off the surface of the wafer. Although high anisotropic surfaces can be obtained, selectivity is often poor. Finally, reactive ion etching (RIE) combines the plasma and sputtering processes. Reactive gases are ionizes through plasma systems, and those ions are accelerated to bombard the wafer surface. Thus, etching is a combination of chemical reaction and momentum transfer.

Two types of etchants are commonly employed in silicon micromachining. Isotropic etchants etch the silicon crystal at the same rate in all directions and can create rounded shapes. Anisotropic etchants etch in a preferred direction. Also known as orientation-dependent or crystallographic

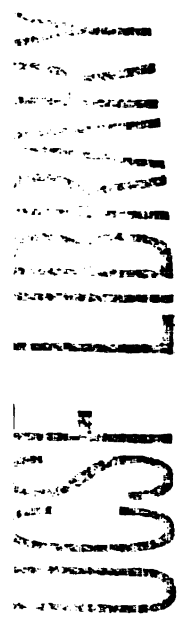
etchants, they can etch at different rates in different directions to form well-defined shapes with sharp edges and corners. The possibility of using new materials for BioMEMS fabrication obviously depends on the availability of proper etchants.

Isotropic etchants are usually composed of mixtures of hydrofluoric, nitric, and acetic acids. These etchants are used to remove silicon from the bulk wafer isotropically (equal etch rates fast in all directions). Because the etchant acts uniformly in all directions, the cavities formed by them are hemispherical in nature.



Anisotropic etchants are key elements in micromachining. They are usually all hot basic solutions such as aqueous potassium hydroxide (KOH), sodium hydroxide (NaOH), and ethylenediamine pyrocatechol (EDP). This is because anisotropic etchants can etch patterns more precisely without undercutting the oxide mask. Thus, an anisotropic etchant can creates complex arrays of structures, placed as close together as the limits of resolution allow. The shape of a hole or pit that is formed by an anisotropic etchant depends on the orientation of the atomic planes in the silicon wafer. The difference in etch rates is due to the different surface energies associated with different crystallographic orientations. Since it is harder to remove an atom from a direction with a high surface energy , the etch rate is in that direction will be lower. The difference in etch rates means that one family of directions will be the limiting directions in the etching of the bulk, leading to a cavity in the bulk that is defined by the crystal planes belonging to the limiting direction's family

of planes. Most anisotropic etchants proceed rapidly in the crystal direction perpendicular to the (110) plane and more slowly in the direction perpendicular to the (100) plane. Etching in the direction perpendicular to the (111) plane is even slower. For both EDP and KOH, the limiting direction is the <111> family of directions, so etching with either of these chemicals gives a cavity that is defined by {111} planes. For example, a square opening oriented along the <110> directions of a <100> wafer, produces a pyramidal pit with {111} side walls. Holes with parallel side walls can be created by using wafers of different orientation such as <110>, where two sets of {111} planes are perpendicular to the surface and not to each other. The differences in etch rate are dependent on the temperature and composition of the etchant, as well as the density of atomic bonds on the particular silicon plane. The size, shape, and orientation of the oxide opening also play a role in determining what type of hole will be formed. Figure 4.4.1 illustrates how different cavities can be defined.



This anisotropic etching technique is often termed bulk micromachining. It is used for backside wafer wet-etching and is a key protocol in BioMEMS fabrication. Through bulk micromachining, key structural elements can be obtained such as channels, orifices, beams, and bridges. As mentioned, the x-y shape of the bulk micromachined cavity depends on the geometry of the opening in the etch mask. . Because of the crystallographic orientation of the wafers used in our experiments ((100) oriented wafers), the cavity walls defined by EDP and KOH etching are at an angle of 54.74° to the surface of the wafer.

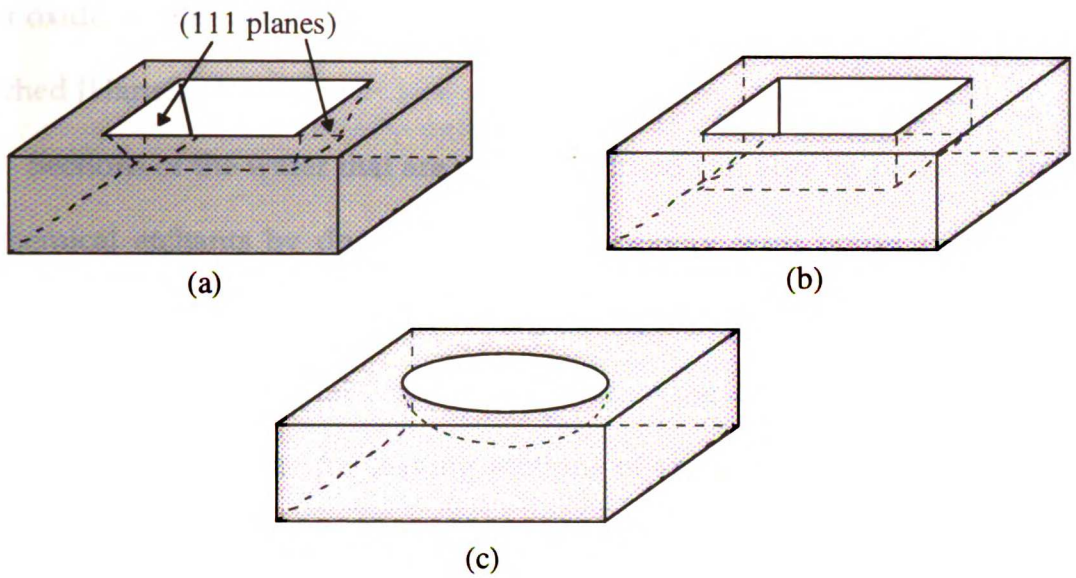


Figure 4.4.1 Etch pits defined by: (a) anisotropic wet etching, (b) anisotropic dry etching, and (c) isotropic wet etching [D. Hansford, 1996]

For both isotropic or anisotropic etchants, one of the most important issues when etching through the entire thickness of the wafer is the selectivity of the etchant (i.e. how large the etch rate ratio is of silicon to the mask material). Because of the large volume being etched, the wafers must be etched for long periods of time. If an etchant is not selective enough, the mask used to define the etched areas may be completely removed and the entire wafer can be etched. One must choose a mask that is thick enough to withstand the etchant, based on its selectivity. For example, EDP has an etch rate ratio of 1:3,300 for silicon to silicon oxide grown by wet oxidation. This means that to use oxide as an etch mask on a normal test wafer (≤550 μm thick), there must be at least 0.17

μm of oxide on the surface to fully protect the areas of the wafer that are not to be etched [Hansford, 1996].

Sections of the wafer that are not to be etched can also be protected from the chemical etchants by doping those areas heavily with boron (to give p^+ doped regions). This doping leads to large compressive stresses in the crystal, which prevents the chemical etchants from removing atoms in those regions. This is a useful technique for leaving sections of single crystal (or polycrystal) as a membrane over an etched section of the wafer for devices.

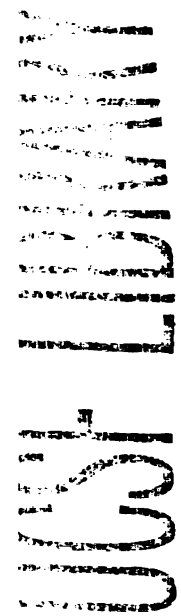
This overview of microfabrication processing will enable one to appreciate the novelty of the technology pursued in the development of the microfabricated biocapsule. Not only are standard bulk and surface micromachining techniques employed, but several creative strategies and fabrication methods are used to realize the needed pore size and configuration of the biocapsule membrane and the overall containment capsule.

5. Immunoisolated Cell Transplant Therapy

> "The potential of artificial cells is limited only by one's imagination. An entirely new horizon is waiting impatiently to be explored..."
>
> - T.M.S Chang

5.1 Concept and History

The transplantation of cells with specific biosecretory function is becoming an increasingly common treatment option for a wide variety of human pathologies. The concept of cell transplant therapy is relatively straightforward, involving the replacement or augmentation of a body's diseased cells with allo- or xenogeneic donor cells in order to restore normal cell secretory function. Living cells transplanted into host organisms can secrete bioactive agents, such as enzymes or hormones, at specific rates governed by cell dose or cellular feedback mechanisms, thus creating living "bioreactors."



Cell transplantation in humans dates back to the early 15th century when patients underwent whole blood transfusions, unaware of the cellular components of blood. Since then, much progress has been made in area of organ transplantation. However, it was only at the beginning of this century, with the development of effective cell isolation and purification strategies, that the transplantation of cells from specific organs became possible [Lanza and Chick, 1996]. By using a combination of mechanical, chemical and enzymatic methods, large quantities of cells could be separated from whole organs while

preserving viability and functionality. Proteolytic enzymes such as collagenase and liberase are commonly used for destroying the architecture of the whole organ while preserving the single cell or functional cell cluster.

The last ten years have seen a great amount of progress in terms of cell transplantation technology. In addition to the widespread use of blood transfusions and bone marrow transplantation, several clinical trials of cell transplantation for the treatment of human diseases are underway. Table 5.1 lists some of the more common applications of cell transplantation therapy.

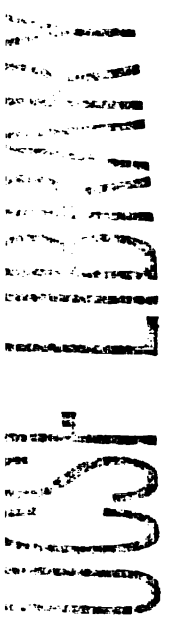
Table 5.1 Applications of Cell Transplant Therapy [Adapted from Inverardi and Ricordi, 1996]

Cell Type	Pathology
Islet of Langerhans	Type I Diabetes Mellitus
Hepatocytes	Liver failure, enzymatic defects
Neural Cells	Parkinson's, Huntington's, Alzheimer's, spinal cord injury
Bone marrow and hemopoietic stem cells	Leukemia, chemotherapy recovery, anemia, immunologic dysfunction
Chondrocytes	Articular Cartilage defects
Myoblasts	Muscular dystrophy
Epidermal	Wound healing, skin lesions
Endothelial	Vascular grafts

Of all cell transplantation, that of bone marrow cells is most established, dating back more than 50 years. The goal of bone marrow transplantation is to provide a new population of self-regenerating multipotent stem cells that are

capable of proliferating and differentiating into mature hemolyphopoietic elements of the blood that have been previously damaged.

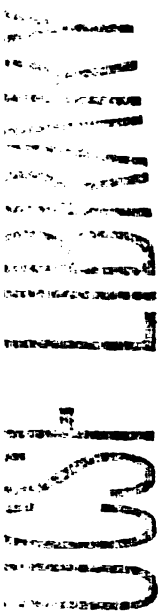
The transplantation of epidermal cells has been researched extensively for the treatment of burn victims. Such cells can be cultured in vitro and subsequently applied to facilitate wound healing and skin regeneration. Recent clinical trials have shown proliferation and differentiation of dermal cells into fully functional epidermal layers suitable for grafting [Hansbrough JF et al., 1992]. Similarly, chondrocyte transplantation has been investigated for cartilage reconstruction. Experiments in rodents have shown that the implantation of chondrocytes within three dimensional matrices promotes cartilage formation in predetermined shapes.



Neural tissue transplantation has received quite a bit of attention during the past decade due to its association with several chronic degenerative diseases such as Parkinson's and Alzheimer's. Beginning in the early 1900s, attempts were made to transplant neural tissue into the CNS. Eventually it was established that neural grafts could survive and integrate into host CNS and that cell transplantation could be used to replace deficient neuronal cell populations. For example, Parkinson's disease could potentially be treated by the transplantation of adrenal medullary neurons from the substantia nigra and Huntington's disease could be reversed by neuronal implants from the nucleus striatus. Likewise, cells from the hypothalamus could be used to correct hypothalamic hormonal secretory defects and chronic pain could be lessened

with transplantation of adrenal medullary chromaffin cells secreting neuroactive substances.

The transplantation of cells from more complex organs such as the liver and pancreas poses a bigger challenge than other kinds of cellular transplantation and has been the subject of intense investigation in a variety of disciplines for the last three decades. The most physiologic way to replace exocrine and endocrine organs is by cell or tissue transplantation. However, it is because of the complexity of both hepatocyte and islet function, architecture, and maintenance that focus has been placed on transplantation approaches involving bioartificial devices. These technologies, involving pancreatic islets, will be described in greater detail in chapter 7.



Although cell transplantation therapy can be used to replace several different organ and tissue systems, similar factors influence the ultimate success of any engraftment. Of course, the implant site is critical in maintaining transplanted cell viability . Long term cell survival will depend on neovascularization and adequate diffusion of nutrients. In addition, the availability of suitable growth factors at the particular site and the absence of harmful compounds such as nitric oxide also affect transplant success.

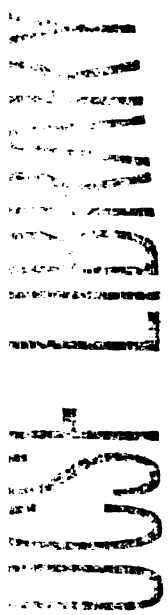
Perhaps the most critical aspect of cell transplantation is that of immune recognition by the host. Allografts, cells and tissue harvested from the same species, are somewhat easier to transplant than xenografts or cells from different species. The mechanisms dictating graft acceptance and rejection will be discussed further in section 5.2. Nevertheless, until recently, the cells could

only be transplanted with concomitant administration of toxic immunosuppressive drugs in order to avoid complete rejection of the donor cells by the host. Moreover, the supply of allografts is limited by the amount of cadaveric tissue available for transplantation. The use of dividing human cell lines or genetically altered human cells with lower immunogenicity could potentially solve this problem, but use of human or cancerous tissue brings up several ethical and legal issues.

Improvements in cell transplantation therapy have been made due to advances in transplant immunology, surgical techniques, immunosuppressive drugs, and organ/cell procurement. Research into cell transplant antigenicity has revealed the role of MHC class I expression in the outcome of grafts. Such knowledge has led to developing animal models with MHC -depleted grafts. Additionally, induction of tolerance to donor-specific cell grafts or interference with costimulatory pathways of immune recognition has been achieved in small animal models.

The use of fetal cells has been explored as a source for transplantation. It has been shown that such cells are less immunogenic and tend to proliferate in vitro as well as differentiate in vivo. Similarly, gene transfection of transplanted cells can potentially modulate immunogenicity prior to transplantation. Manipulation of cell grafts *in vitro* prior to transplantation through UV-irradiation has also been somewhat effective in preventing acute rejection.

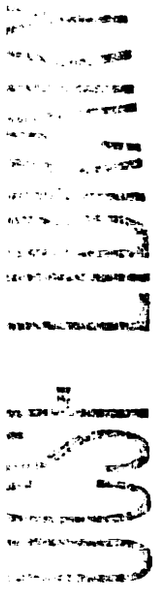
Most immunosuppressive drugs have the disadvantage of being non specific, thus placing the patient at high risk for infection. Furthermore, many immunosuppressive drugs are aimed at ceasing the proliferation of activated lymphocytes. However, other proliferating cells such as epithelial or bone marrow hematopoietic stem cells are often deleteriously affected. Cyclosporin A is an agent often used in heart, kidney, pancreatic, liver, or bone marrow transplantation because of its potent immunosuppressive effects. Essentially it works by blocking activation of resting T cells by inhibiting the transcription of genes encoding IL-2 and the IL-2 receptor, both of which are essential for activation.



In recent years, attention has turned to developing alternative ways to immunoprotect transplanted cells. One of the most promising approaches to graft immunoprotection is cell encapsulation technology, establishing the foundations for immunoisolated cell transplant therapy. Early investigations into immunoisolation therapy date back to over sixty years ago with attempts to create a plastic housing for organs [Biseglie, 1933]. However, it was Chang et al. (1964) who first proposed microencapsulation of biological substances for organ replacement in a pioneering work entitled "Artificial Cells." He wrote "The 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." By 1965, Chang was able to demonstrate the feasibility of using artificial cell encapsulation for diabetes,

liver failure, and a variety of drug delivery applications. Serious interest in this therapeutic approach grew in the late 1970s and early 80s when investigators such as Chick et al. (1977) and Lim and Sun (1980) successfully treated chemically-induced diabetic rodents using immunoisolation islet grafts. Since then, immunoisolation technology for cell immunoisolation has been investigated vigorously. The same basic principles of cell immunoisolation have been applied to the treatment of several diseases as will be described in section 5.3.

Regardless of the nature of the transplanted cells, the same basic principles are applied in all immunoisolative cell transplant therapies. Cells are placed in an immunoisolation device and are either free floating within a capsule or supported on a three dimensional matrix or internal scaffold. The most critical aspect of the immunoisolation device is the immunoisolation membrane, often thought of as the enabling technology of cell encapsulation.



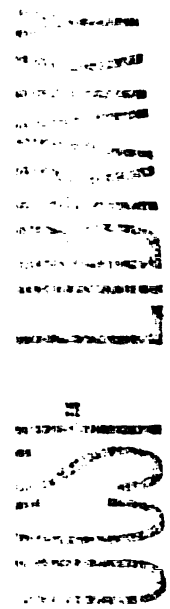
5.2 Transplant Immunology

In order to develop effective immunoisolation strategies, an adequate understanding of the complex processes occurring in the active immune system is required. The immune system is the body's main line of defense against invasion by foreign substances, including organ or cellular transplants. In most cases, the term "foreign" means anything not recognized by the host organism's immune system as being its own. The immune system evolved

based on the need to protect the body from invasion by pathogenic microorganisms and resist disease.

Table 5.2 Issues in Encapsulated Cell Transplant Therapy

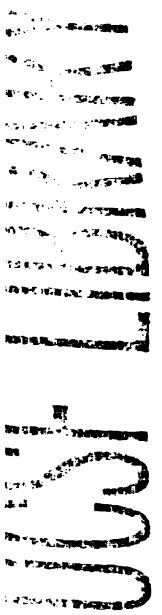
• Technology	Macrocapsules Microcapsules Microfabricated	Polymer or Inorganic
• Location	Vascular	Intravascular Perivascular
	Portal	Intraperitoneal Intrahepatic Intrasplenic
	Systemic	Subcutaneous Intramuscular
• Cell Source	Human Animal Tumor cell lines Genetically engineered	Fetal or Adult
• Effectiveness	Mechanical stability Chemical stability Biocompatibility Diffusion kinetics Immunoprotection	



Understanding the mechanisms of transplant rejection and acceptance are central to the development of an effective immunoisolation device. The degree of immune response to a particular transplant depends on the type of graft. An autograft is self-tissue transferred from one site of the body to another in the same host. Allografts are grafts between genetically different members of the same species. However, since the need for organs is growing steadily and there is a shortage of human donors, more emphasis has been placed on utilizing

tissue from discordant species. Such use of animal organs in lieu of human organs is called xenotransplantation. Xenografts, obviously, have the greatest genetic differences and therefore, elicit the greatest graft rejection. Successful transplantation of discordant xenografts has remained an elusive goal with several challenges waiting to be overcome.

Tissues that are antigenetically similar are termed histocompatible and do not exhibit graft rejection. Those that are not antigenetically similar are histoincompatible and induce a strong immune response leading to graft rejection. The genetic loci responsible for allograft rejection are located within the major histocompatibility complex (MHC). The exchange of grafts between allogeneic individuals leads to rejection, regardless of whether both are homozygous or heterozygous for the histocompatibility loci distinguishing the two grafts. Rejection is more rapid the more different the genes in the major histocompatibility loci.



It was J. Haldane in 1933 who first suggested that the basis for graft rejection was immunological. During World War II, Peter Medwar confirmed this theory by establishing that graft rejection results in a state of antigen-specific memory in which a subsequent graft is rejected more rapidly than the first. Furthermore, he showed that this rejection happened regardless of the transplant site and therefore, was systemic in nature.

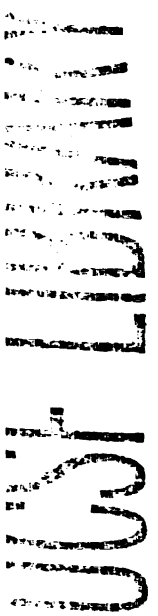
The time of allograft rejection is dependent of the tissue type. Generally, first set rejection takes place within a week after transplantation and the transplant becomes infiltrated with inflammatory cells. By ten days, there is

decreased vascularization and visible necrosis. In some instances, hyperacute rejection can occur within the first 24 hours after transplantation. This is triggered by preexisting host serum antibodies specific for graft antigens. Antigen-antibody complexes activate the complement system (discussed in detail later), leading to intense inflammatory cell infiltration and avascularization.

Allograft rejection takes place primarily through a cell mediated immune response to alloantigens on the surface of the graft. The allogeneic reaction can be divided into the recognition and effector phases, involving both a specific and non-specific inflammatory response [Morris PJ, 1996]. In a conventional foreign body response, the antigen is first recognized and process by an antigen presenting cell (APC) which is a dendritic cell (DC) in tissues. The processed antigen or peptide is then presented by the APC in conjunction with the class II antigen of the major histocompatibility complex (MHC) to the T-helper subset of lymphocytes. This coupling of antigen together with the class II MHC signals an activation and proliferation of the T-helper cell population.

The effector stage involves the release of specialized cytokines by the activated T-cells. These cytokines have many functions, including activation of macrophages, generation of cytotoxic T lymphocytes, and signaling to B lymphocytes to produce antibodies to the antigen. In addition to indirect recognition as described above, an allograft can be recognized directly. In this

case, the foreign MHC alloantigen on the allograft is recognized directly by the T-helper cell of the recipient, leading to its activation.



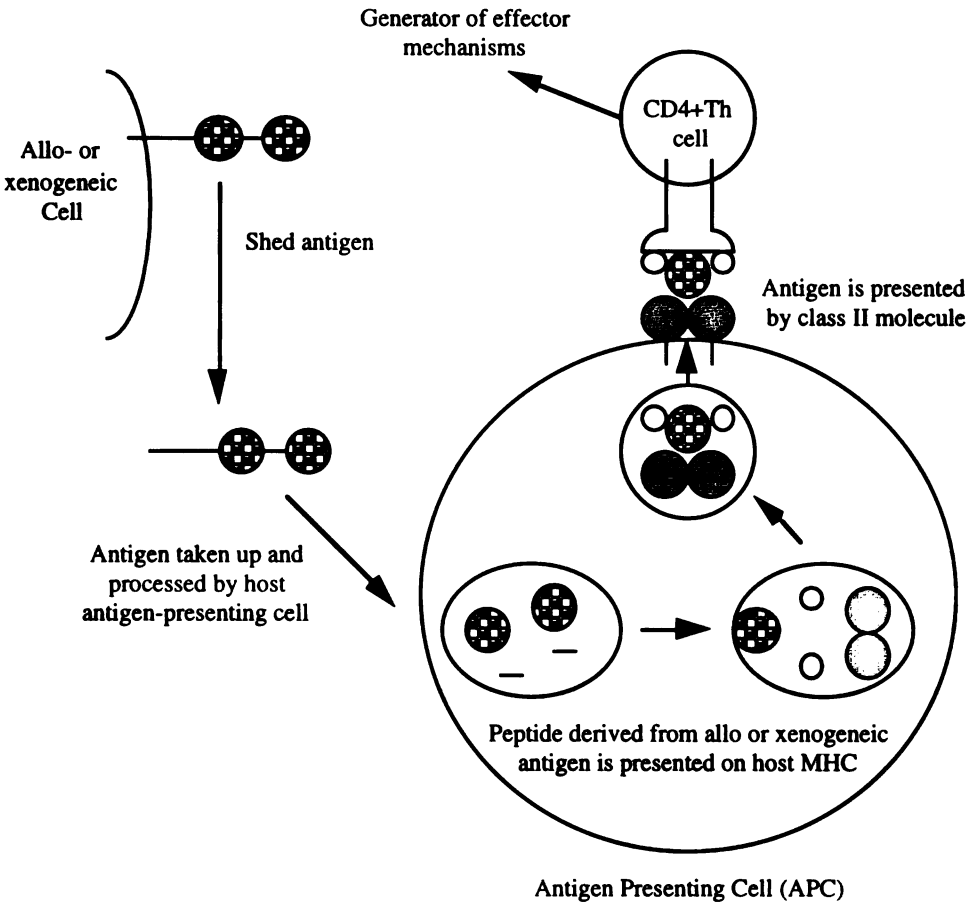


Figure 5.1 Indirect recognition: Cell antigens shed from grafts are processed and presented to CD4+ Th cells in association with host MHC class II by antigen presenting cells (APCs).

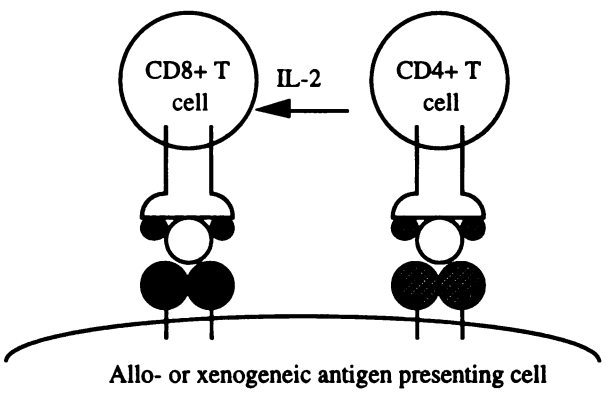


Figure 5.2 Direct recognition: T cells recognize MHC directly on the surface of the donor APC without need for antigen processing / presentation on host APC

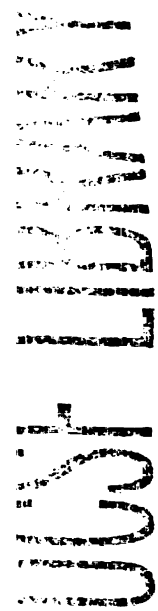
In some organ and tissue grafts such as the pancreatic islets, a population of donor APCs called passenger leukocytes is often present in the graft [Kuby, 1996]. The leukocytes, with allogeneic alloantigens, can migrate from the graft to the host lymph nodes and stimulate immune activation of T lymphocytes in the lymph node.

After the recognition or afferent phase of the immune response, the effector phase ensues. There is both a specific cellular response and a non-specific humoral one. Cytotoxic T lymphocytes are involved in the cellular response and can kill the target alloantigen following cell-cell contact. The initial humoral response is dominated by the B cells. B cells, in response to an invasion of a foreign substance, produce a globular, circulating glycoprotein termed an antibody which recognizes and attaches to the foreign substance. The foreign substance or antigen interacts in a specific way with the antibody to trigger a series of events leading to the ultimate destruction and/or elimination of the substance from the host system. This immune response is called humoral immunity because the active agent (the antibody) is found freely circulating in the body's "humor", otherwise know as the blood and lymph system. The humoral immune response is directed at a wide range of foreign invaders, both cellular and molecular.

5.2.1 Humoral Immunity

Two main classes of antibodies, IgM and IgG, are involved in the allogeneic humoral response. These antibodies constitute the majority of complement activating immunoglobulins in the plasma and have been the

focus of most transplantation studies. The allo-antibody binds to target epitopes of the allograft MHC antigen and activates the complement pathway leading to cell destruction. It is critical to examine the structure and function of antibodies in order to gain a better understanding of their importance in foreign body responses. Antibodies are produced by the B cell, a white blood cell type. Antibodies generally have the same basic four polypeptide chain immunoglobulin structure consisting of two light (L) chains and two heavy (H) chains held together by disulfide bonds. This unit has two antigen combining sites at the NH2 terminal ends of adjacent heavy and light chains.



Thus the antibody has both a structural and functional element: the H2L2 configuration coupled to with an ability to interact selectively with a particular antigen. Humans, in particular, possess a large structural and functional repertoire of immunoglobulins. These immunoglobulins are broken down into five distinct classes which are distinguished on the basis of their primary structure. The two immunoglobulin classes that we will consider further are IgG and IgM, both of which are involved in foreign body reactions.

IgG is the principal immunoglobulin in human serum. Although only small amounts of IgG are produced in a primary immune response, it is the main type of antibody produced in a secondary reaction upon reintroduction of the antigen. It accounts for nearly 80% of the total serum immunoglobulin, providing the main defense against bacterial infection. The IgM immunoglobulin has a pentameric structure held together by interchain disulfide bonds linking the C-terminal portions of adjacent monomeric chains.

Because of its large size, IgM is usually found circulating in the blood, rather than in tissue spaces. The exact dimensions of the IgG antibody are still unclear. Studies using atomic force microscopy revealed individual IgG molecules with lateral dimensions of about 25-40 nm [Zhang et al., 1997].

The initiation of the complement pathway is the main effector mechanism of the humoral immune system. It is an enzymatic cascade that is comprised of over 20 proteins that interact in a regulated manner. Although there are two complement pathways, classical and alternative, both lead to formation of a membrane attach complex (MAC), which lysis the target cell. The classical pathway begins with the interaction of one IgM antibody or two adjacent IgG antibodies with antigenic epitopes. This interaction induces a conformational change in the antibody and fixes the first complement molecule, C1. In contrast, the alternative pathway is initiated by foreign cell surfaces such as some immune complexes or Gram negative bacteria.

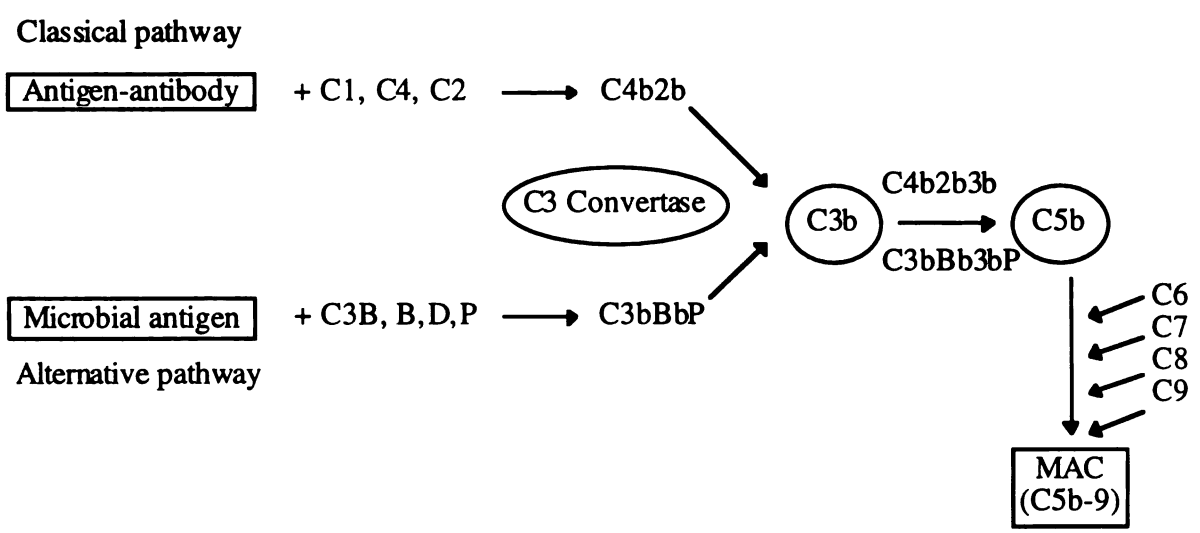


Figure 5.3 Classical and alternative pathways initiated in complement cascade

5.2.2 *Xenogeneic Response*

The xenogeneic immune response has several distinguishing features from the alloresponse. The cell-mediated immune response is usually active in xenorejection. For example, a T cell recognizes and directly binds to the foreign antigen, leading to its elimination. These T cells are different from antibody producing B cells in that they are usually directed towards antigens directly presented on the surface of pathologically altered cells.

In addition, xenoantigens are usually recognized through the indirect pathway, where the xenoantigen is process by host APCs and presented to helper T lymphocytes. Therefore, even if cell-cell contact and/or complement-mediated humoral cell destruction is prevented, one still must contend with indirect xeno-antigen presentation and processing. This makes xenograft immunoisolation a much greater challenge than allografts.

The immunopathogenesis of xenograft rejection is a complex phenomena worth considering. The first response is that of hyperacute rejection which is primarily characterized by interstitial hemorrhage and diffuse thrombosis [Parker, 1996]. Initiated immediately upon reperfusion of the organ xenograft, this process can destroy a graft within minutes to hours. Hyperacute rejection most often occurs in discordant rather than concordant cross species transplantation, and it is believed to have a genetic basis, reflecting the phylogenetic distance between donor and recipient [Platt JL et al., 1991].

Porcine organs seem to be especially susceptible to hyperacute rejection by humans. This is speculated to be caused by two factors: 1) the finding of xenoreactive natural antibodies of the recipient to antigens on endothelial cells in the graft and 2) the incompatibility of complement regulatory proteins in the transplant with the complement system of the recipient, stimulating activation of the recipient's complement system.

There is mounting evidence that the development of hyperacute rejection depends on the activation of complement. However, it is unclear the exact mechanism by which complement is activated in xenogeneic tissue. If hyperacute rejection can be successfully overcome, then there is a second rejection type that must be examined. In a period of days to weeks, a xenograft is subject to acute vascular xenograft rejection, otherwise termed delayed xenograft rejection [Leventhal JR et al., 1993]. This reaction is characterized by diffuse intravascular coagulation and interstitial inflammation. The onset of delayed xenograft rejection seems to correspond to an increase in xenoreactive natural antibodies [Cotterell et al., 1995]. These antibodies may destroy the xenograft by several mechanisms including activating the complement pathway, delivering signals to activate endothelial cells [Lampunani MG et al., 1991], or serving as a ligand for cytotoxic cell receptors [Inverardi et al., 1992]. Delayed xenograft rejection is further characterized by donor-organ endothelial cell activation and the infiltration of host monocytes and natural killer cells into the graft [Bach et al., 1996]. Together, these cells promote graft inflammation and thrombosis through coagulation and platelet aggregation.

There is much anticipation over the use of xenografts in cell therapy. Examples such as the "Baby Fae" heart transplant and baboon to human liver transplants demonstrate the potential therapeutic value of xenografts. Therefore, the development of effective engineering strategies, such as cell encapsulation for immunoisolation, in combination with extensive research in transplantation immunology are even more critical as we move into the next century.

5.3 Applications of Immunoisolative Cell Therapy

The application of immunoisolated cell transplant therapy has become widespread, ranging from diabetes [Lacy et al., 1991, Lanza et al., 1992] to disorders of the central nervous system [Aebischer et al., 1988, Winn et al., 1992]. Table 5.3 summarizes some of more common applications and their development status.

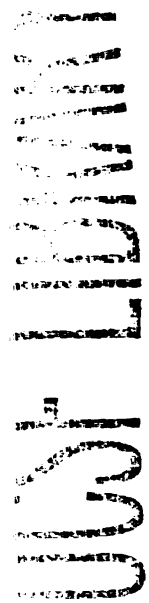
This technology applied to the treatment of chronic pain [Sagen et al., 1993] has met with great promise and is likely to be the first application to be successful in human clinical trials with xenogeneic cells and a full-sized device. Both large and small animal studies by Sagen and Aebischer [Sagen, 1992; Sagen et al., 1993] show long term release of catecholamines from rodent adrenal chromaffin cells. More recently, immunoisolative cell therapy has been applied to the blood components and erythropoietin [Liu et al., 1993, Koo and Chang, 1993].

Table 5.3 Status of some applications of Immunoisolative Cell Therapy [Adapted from Lysaught et al., 1994]

Application	Development Status	Cells	Secretory products	Reference
Alzheimer's	Rodent studies	Rodent fibroblastic cell lines	Recombinant nerve growth factor	Hoffman et al., 1993
Anemia	In vitro studies	Kidney cell isolates	Erythropoeitin	Koo and Chang, 1993
Chronic Pain	Large animal xenograft studies	Bovine adrenal chromaffin cells	Catecholamines	Sagan, 1992
Diabetes	Canine and small animal allografts	Islets	Insulin	Soon-Shiong et al., 1993
Hemophilia B	Canine	Rodent fibroblastic cell lines	Factor IX	Liu et al., 1993
Parkinson's	Subhuman primates	PC-12 cell line	Dopamine	Aebischer et al., 1991a

6. Overview of Type I Diabetes Mellitus

Diabetes mellitus in humans has become a significant national health problem that is associated with such complications as blindness, kidney disease, heart disease, and stroke[Draznin and LeRoith, 1994]. Type I diabetes mellitus in humans is an autoimmune disorder in which the insulin-secreting endocrine pancreas is destroyed by an immunological response. The individual then becomes hyperglycemic (blood sugar level > 130 mg/dl) and would die without exogenous insulin. Although several therapeutic alternatives to diabetes treatment have been explored over the past two decades, there is still no clear and easy way to prevent long term diabetic complications. Rather than only replacing or augmenting the missing hormone, insulin, replacing the diseased insulin secreting endocrine tissue of the pancreas with healthy tissue is ideal. This approach would provide insulin delivery at regulated physiological doses which would prevent many of the pathological complications of diabetes.



6.1 Physiology of the Pancreas

The pancreas is a slender lobulated gland located in the upper abdominal space framed by the duodenum and spleen. Although most of the pancreas is an exocrine glad which secretes proteolytic and lipolytic enzymes, endocrine tissue is heterogeneously interspersed throughout the gland. The endocrine pancreas comprises 1 to 2 percent of the pancreatic mass and is composed of 1 to 2 million separate and individually innervated cell clusters known as islets of Langerhans. Each islet is supplied with blood through the pancreatic artery and

drains into the portal vein, thus delivering the output of pancreatic hormones to the liver. In their native environment, islets are highly vascularized and account for approximately 10% of the pancreatic blood flow. The islet blood flow is directed from the islet center towards the periphery [Cooperstein and Watkins, 1981].

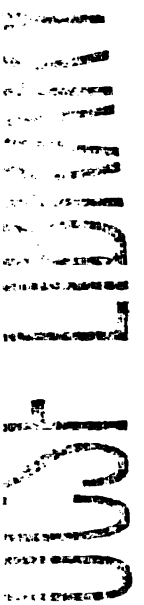
The pancreatic islet is made up of several endocrine cell types typically arranged as a Beta-cell core surrounded by a mantle of alpha (α) cells, delta (δ) cells and pancreatic polypeptide (pp) cells. The alpha cells account for 20% of the islet and secreted glucagon, a fuel-mobilizing catabolic hormone which stimulates the liver to release energy-rich molecules such are glucose, aceto-acetic, and b-hydroxybutyric acids, into the circulation. Beta cells which secrete insulin comprise 60-75 % of the islet. Insulin is a fuel-storing hormone anabolic hormone that promotes sequestration of carbohydrate, protein, and fat in the liver, muscle, and adipose tissue. Delta cells, scattered amongst the alpha and beta cells, produce somatostatin which is thought to "brake" the secretion of insulin and digestive enzymes in order to slow down the absorption of food. The function of other cells such as the pp cells has not been clearly identified, although there is some evidence that these cells function in the secretion of gastrin.

6.1.1 Insulin

Insulin, a 6 kilodalton protein, plays a critical role in integrating metabolism. In contrast to glucagon, the production of insulin is essential for survival. The secretion of insulin by the pancreatic beta cell is stimulated by

both glucose and the parasympathetic nervous system. Insulin is known to stimulate glycogen synthesis in both muscle and liver and suppress gluconeogenesis by the liver. It also increases glycolysis by the liver which increases the synthesis of fatty acids. Insulin promotes the uptake of glucose into muscle and adipose cells as well as promotes amino acid and protein metabolism. Although it was the first protein to be crystallized, sequenced, and synthesized by chemical and recombinant DNA there are still several aspects of insulin synthesis and secretion that remain unclear.

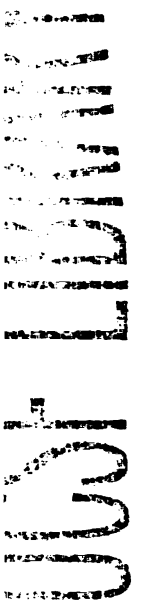
Insulin consists of two dissimilar chains of amino acids, A and B. The chains are held together by disulfide bonds which connect positions A-7 to B-7 and A-20 to B-19. The A chain also has an internal disulfide bond bridging position A-6 to A-11 that forms a six amino acid loop. All mammalian insulins have the same disulfide linkages, suggesting that the secondary and tertiary structure of this protein is essential to its function. The isoelectric point of this acidic globular protein in 5.3, meaning it is slightly negative at physiological pH.



6.2 Islet Cell Physiology : Insulin synthesis

The pathway of insulin biosynthesis begins in the beta cell with the synthesis of a precursor, called preproinsulin. The synthesis of preproinsulin, as well as all proteins, occurs on cell ribosomes. A molecule of messenger RNA (mRNA) must be copied from the DNA that encodes the specific protein. At the same time, in the cytoplasm, each of the 20 amino acids from which the protein is to be built is attached to its specific transfer RNA (tRNA) molecule. These

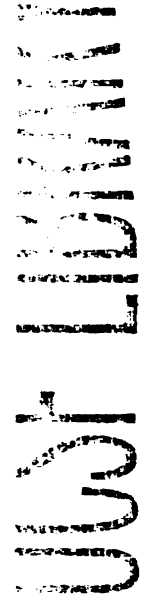
components all come together in the cytoplasm to form a functioning ribosome. The molecule of mRNA then moves stepwise through the ribosome where the DNA sequence is translated into a corresponding sequence of amino acids to produce the protein chain. Human preproinsulin mRNA encodes a 11,500 dalton protein that has a 24 amino-acid, hydrophobic NH_4-terminal signal peptide. Signal peptides are thought to regulate protein sorting and secretion. The signal peptide sequence on a secretory protein is usually located at the NH_2 terminus and has a 15-30 amino acid sequence with a net positive charge and a small side chain at the cleavage site. While translation of *cytosolic* proteins starts and ends on cytosolic ribosomes, translation of *secretory* proteins is diverted. A signal recognition particle recognizes the signal peptide and serves as a docking site for secretory proteins on the cytosolic dies of the rough endoplasmic reticulum (RER) [Rothman and Orci, 1996]. Once docking is complete, the signal recognition particle leaves and translation finishes in the RER. There is unidirectional and cotranslational passage of preproinsulin through the membrane of the RER into the cisternal space. This is the only time that the protein crosses a hydrophobic membrane carrier.



Although the order of preproinsulin post-transitional events is not precisely known, it generally has the following sequence. After the synthesis of preproinsulin, it is rapidly cleaved to proinsulin during or shortly after its tranlocation across the RER membrane. The signal peptide is removed and a series of post-translational proteolytic modifications by peptidases occur. Within the lumen of the RER, proinsulin quickly folds to allow formation of

the three site-specific disulfide bonds. It is these disulfide bonds which stabilize the specific folding of the protein molecule. Preproinsulin has a half life of only 1-2 minutes before it is converted to proinsulin.

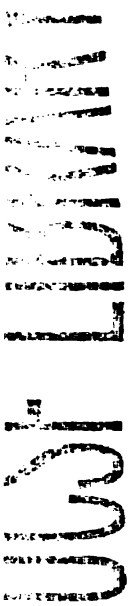
Proinsulin is a 9000 dalton single peptide, which includes the entire insulin dipeptide and a connecting peptide (C-peptide). This c-peptide is usually linked to the -COOH terminus of insulin's B chain by an -arg-arg- linkage and to the A chain NH_2 terminus by an -lys-arg- link. Proinsulin is the direct precursor to insulin and is, itself, a functional hormone. It is transported from the RER, through the beta cell, to a site of additional processing in the Golgi apparatus. Protein precurors enter the cis side of the Golgi apparatus and are gradually transported to the trans side. The proteins exit the RER in transport vesicles which bud from transitional elements of the RER membrane and are delivered to the cis region of the Golgi complex and on through the Golgi cisternae by repeated cycles of vesicle budding and fusion [Rothman and Wieland , 1996].



Once in the Golgi apparatus, the proinsulin is packaged into secretory granules. It is in these granules that most of the final conversion to insulin occurs. Conversion of proinsulin to insulin requires enzymes which first cleave bonds separating insulin's A and B chains. Then basic amino acids from newly exposed NH_2-termini on the C-peptide and A chain and COOH-termini are removed. This results in an equimolar production of insulin (A and B chains connected by disulfide bonds) and C-peptide. Proteolytic cleavage is an essential and irreversible step in the insulin assembly process. For example, if

part of the proinsulin chain is excised before complete processing, there is an irretrievable loss of the information needed for the protein to fold spontaneously into its normal conformation.

The cellular transport of proinsulin from site of production to site of cleavage takes 15 -30 minutes. There are at least two types of secretory granules containing insulin, some of which have a dense core and others, located closer to the trans Golgi, which are clathrin coated and lack a dense core. Newly formed clathrin coated granules have a neutral internal pH environment whereas mature granules are relatively acidic. Since mature granules contain mainly insulin compared to newly formed granules which are rich in proinsulin, acidification seems to by an important event in the initiation of prohormone conversion [Orci, 1986]. These granules have a pH of 5.0-5.5 which corresponds to the isoelectric point of insulin. This environment promotes the self-association of insulin into crystals which are ready for secretion. Insulin in secretory granules is extremely concentrated (10-2 M) and can provide a 5 day supply for a human.



In the final step of protein secretion, secretory granules fuse with the plasma membrane and release their contents to the extracellular space in a process called exocytosis. In some cases, proinsulin conversion to insulin is not complete before exocytosis, leading to 2-5% proinsulin secretion. Proinsulin remains intact in circulation and often competes with insulin for functional binding sites. The acute biosynthesis of insulin is essential to maintain homeostasis. Insulin is released in proportion to needs and therefore, glucose

levels do not fluctuate outside of a narrow margin. The response time of pancreatic islets to an increase in blood glucose is extremely rapid - less than one minute for the initial burst in insulin release primarily from intracellular stores. This is followed in a diphasic manner by a gradual increase in insulin secretion to appropriate levels dictated by the intensity and duration of the stimulus. Within 5 minutes of glucose stimulation, the biosynthesis of insulin precursors is augmented 5 to 10 fold. Insulin deficiency leads to a disease called diabetes mellitus which is one of the most common endocrine diseases in the world, affecting as many as 5% of the U.S. population.

6.3 Pathology of Diabetes

In order to begin developing more effective treatment options for diabetics, the pathology of the disease must be fully understood. Diabetes is a chronic systemic disease resulting from either insufficient production of insulin (Type I) or ineffectiveness of the available insulin (Type II). In both cases, the patient has a rise in blood glucose levels or hyperglycemia which can lead to microvascular complications and neuropathies of the autonomic, peripheral and central nervous systems. Type I diabetes is the more severe form of the disease and is the target of cellular encapsulation therapy.

In Type I diabetes, endogenous insulin function is almost completely absent and exogenous insulin is required for survival. Type I Diabetes is representative of classic autoimmune diseases in which lymphocytes

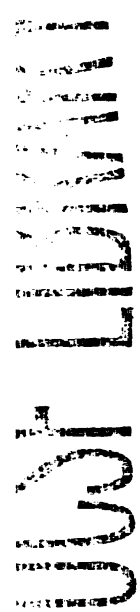
(immunocytes) are activated in response to self-antigens presented on cellular substructures within a tissue that serves a vital physiologic function. The autoimmune process can develop from defects in the selection or expansion of peripheral lymphocytes, abnormal immune regulatory pathways, unusual presentation of autoantigens, or dysfunction of target organs and receptors [Homo-Delarche and Boitard, 1996]. However, the exact events, whether environmental or genetic, that trigger autoimmunity still remain unclear.

Taking a closer look at the molecular mechanisms at work in Type I diabetes, it is seen that the disease is caused by autoimmune destruction of the beta cells of the pancreatic islet, which ultimately leads to loss of insulin secretory function. Generally, the disease presents itself in those whom are genetically predisposed. The inappropriate response to self antigens can involve either the humoral or cell-mediated branch of the immune system. Autoimmune beta cell destruction may progress over a number of years before the disease is overtly expressed. Therefore, the pathogenic development of Type I diabetes is often divided into stages which include: genetic predisposition, overt immunologic abnormalities, progressive loss of insulin release, and overt diabetes [Colman et al., 1989].

There are several histopathologic abnormalities due to cell mediated immunity that are present in type I diabetes. For instance, peri- and intra-islet lymphocyte infiltrate (insulitis) is commonly found in patients within one year of diagnosis of Type I diabetes. However, insulitis seems to only affect the insulin-secreting beta cells of the pancreas. T-cells, B-cells, and macrophages

are also involved in the inflammatory response to beta cells. The disease progression is specific, ultimately resulting in a collapsed alpha cell islet. Additionally, an increased sensitization to pancreatic antigens is observed as well as defective immunoregulation and altered ratios of monoclonally defined T cell subsets.

Islet destruction may result from either an indirect or direct action of the immune system towards beta cells [Calcinaro, Wegmann, and Lafferty, 1994]. For the indirect immune reaction, T-cells interact with antigen presenting cells (APCs) which present islet associated (Ia) antigens. This APC-T-cell contact results in the production of lymphokines by CD4 T-cells , the activation of other inflammatory cells, and their release of cytokines and oxygen free radicals which destroy the beta cells. In indirect islet destruction, both allogeneic and xenogeneic islets are equally susceptible to attack. The direct immune activation happens when T-cells interact with cell surface antigens on the beta cells themselves. It has been found that Type I diabetic patients have markedly increased levels of islet cell antibody and T cells bearing the surface Ia antigen.

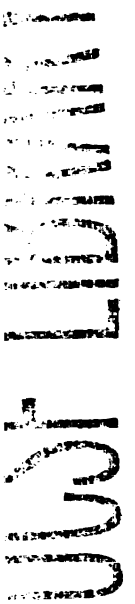


6.4 Clinical Therapies for Diabetes Management

Conventional diabetic therapy includes multiple insulin injections and daily dietary monitoring. Since the early 1980's, self-monitoring of blood glucose (SMBG) has been used to determine if the blood sugar levels of a person with insulin-dependent diabetes mellitus are too high or too low. There

are several instruments available for self-monitoring of blood glucose. The measurement helps you monitor your diabetes control to determine if adjustments in diet, insulin, or exercise are needed. However, all of these monitoring techniques require full patient cooperation and motivation. In addition, the margin of error in patient monitoring can be high.

The open loop insulin pump is one option for treating diabetes in the long-term clinical setting. The pump provides a programmable subcutaneous infusing of insulin, and can be external or implantable. It tries to imitate the normal patterns of insulin secretion by providing regular doses of insulin on a specified basis [Westphal and Goetz, 1990]. An insulin pump is made up of a pump reservoir (like a regular syringe, but bigger) filled with insulin, a small battery operated pump and a computer chip that allows the user to control exactly how much insulin the pump delivers. The pump is intended to be used continuously and delivers insulin 24 hours a day according to a programmed plan unique to each pump wearer.



However, the pump is not automatic. The user still has to decide how much insulin will be given. In addition to the overall inconvenience of using an open loop pump, there are several risks associated with its use. Since these systems do not make use of blood glucose information, there cannot reliably provide tight blood glucose control. Also, there are often several mechanical failures such as tubing leakage or obstructions associated with pump components.

The research and development into convenient and bloodless glucose measurements has led to an expanding diversity of measurement techniques. Several products are under development that use the techniques of iontophoresis, ultrasound or chemicals to draw the glucose through the skin. For example, work is being done on a fluid absorbing transdermal patch that will chemically enhance the transport of glucose [Azimi, 1995]. These patches will develop color and can be removed after five or ten minutes to be read on a device similar to a typical blood glucose meter.

The method of iontophoresis utilizes low levels of electricity across two points on the skin surface to enhance the transport of both charged and neutral molecules, and is being investigated for transcutaneous drug transport. It's been shown that the passage of a small current across the skin will enhance the transport of glucose to the skin surface [Rao et al., 1993; Tamada et al., 1995].

Nonetheless, there are limitations to this method in that the efficiency of glucose extraction by reverse iontophoresis is not the same in every individual and may require specific calibration for each person. This would mean that the patient would need to perform multiple fingersticks over a broad range of glucose values to recalibrate and thus is not significantly advantageous to conventional finger sticking.

A recent development called the GlucoWatch™ uses the reverse iontophoresis (electro-osmosis) technique [Azimi,1995]. The device, worn like a wrist watch, has a disposable pad on its bottom into which glucose is extracted through the skin. The glucose is then reacted with glucose oxidase

and detected through an electrochemical reaction. There are two skin conductivity electrodes on the watch to determine excess moisture and to shut the system down. There is also a thermistor to monitor skin temperature. However, glucose oxidase degrades with both heat and water interaction. It is most stable at low pH (pH 2-3 and this is not necessarily compatible with long term skin exposure. In addition, the glucose sensor must operate continuously, measuring very low concentrations of glucose with minimal response to other electrochemically active substances. The use of unbound glucose oxidase in the pads which are continuously in contact with the skin may also be problematic since glucose oxidase is highly immunogenic.

Recently, focus has been placed on the development of implantable sensors that are imbedded into the interstitial space. The most common implanted sensor is an enzymatic amperometric glucose sensor placed into the subcutaneous fat where glucose is anaerobically oxidized or reduced with the involvement of an enzyme and redox mediator. With implanted electrochemical sensors, the measurement technique is taken to the fluid location instead of the fluid being extracted from the body. When glucose reacts with glucose oxidase on the electrode there is an enzyme catalyzed oxidation of glucose, and electrons are transferred onto the electrode surface to be read as a current. This current is proportional to the glucose concentration in the test sample [Gough and Armour, 1995].

Implanted electrochemical sensors have the advantage of true continuous sensing in the interstitial space, and therefore, can be used in a

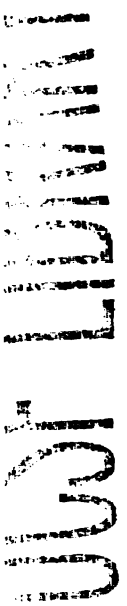
closed loop feedback system for diabetic patients. However they have several major disadvantages. First, the implanted sensor forms a fibrotic layer which results in a barrier between the implant and the blood vascular system. This collagen-rich encapsulation layer around the sensor will increase the glucose mass diffusion time and must be considered when calculating the real-time blood glucose concentration.

An amperometric sensor requires a voltage potential to detect glucose, and often detects other compounds present in the interstitial fluid. Thus, frequent recalibration for long term operation may be required. The electrode chemistry can be easily destroyed by environmental conditions. All implanted medical sensors must be sterilized and this can impair enzyme activity on the sensor.

As has been shown, conventional diabetes therapy is still far from effective in preventing long term clinical complications. Therefore, attention has turned to alternative therapies for the treatment of diabetes. Some alternatives include implantable closed loop insulin pumps, synthetic insulin releasing cells, and whole pancreas transplantations. One of the most promising alternatives to insulin injections is the transplantation of microencapsulated islets of Langerhans.

Transplantation of insulin-producing cells of pancreatic tissue seems to be the ideal method to achieve perfect glycemic control in insulin-dependent diabetic patients for a variety of reasons, especially since physiologic modulation of blood sugar levels is automatically achieved. Pancreatic tissue

transplantation has been proven to be a viable treatment option for insulin-dependent diabetes mellitus. Because of this, the use of pancreatic tissue replacement to normalize sugars and prevent development of diabetes complications is a major goal. However, many problems need to be overcome especially the need to chronically immunosuppress recipients in order to control graft rejection and prevent recurrence of disease in the graft. Currently, nonspecific and relatively dangerous forms of immunosuppression are being used. However, if the use of immunosuppression can be minimized or even avoided and a state of immunological tolerance achieved, recent onset diabetics could be effectively transplanted.



7. The Bioartificial Pancreas

7.1 Concept

The purpose of pancreatic transplantation either as a whole organ or isolated islets is to improve the quality of life for the diabetic patient and hopefully prevent complications associated with long term insulin dependent diabetes. Although whole pancreas transplantation has been more successful than islet grafts, it requires a more complicated surgical procedure and is subject to the availability of whole organs. Islet transplantation, on the other hand, is simpler for the recipient but is more subject to immune rejection [London NJM et al., 1994]. Thus, generalized immunosuppression is required for these transplants and is often restricted to those patients with advanced diabetic nephropathy already on an immunosuppressive regimen for kidney transplants.

As organ and cellular transplantation becomes limited due to tissue unavailability and the need for chronic immunosuppression, researchers have been looking towards new ways of replacing natural organs. One such approach involves what are known as bioartificial or hybrid artificial organs. These represent combinations of both organ transplantation and organ substitution. A hybrid organ combines artificial materials with natural tissues that behave like the organ from which they were taken. It can be used when researchers are unable to artificially duplicate a natural organ's function.

As was previously discussed, the best approach to Type I diabetes treatment is one which restores or reestablishes the body's insulin-glucose feedback system. Inevitably, the therapy will involve either the manipulation or replacement of the insulin-secreting beta cells as shown in Table 7.1.1. The bioartificial pancreas utilizes this concept of combining living tissue with

synthetic components to restore physiologic function. The hybrid pancreas usually consists of live pancreatic cells, the islets of Langerhans, harvested from the organ and subsequently surrounded by a semi-permeable membrane that serves to immunoprotect the cells. This membrane permits the passage of glucose and insulin to and from the pancreatic cells but blocks components of the immune system that can cause rejection.

Table 7.1.1 Cellular approaches to Diabetes Treatment

Beta Cell Manipulation	Beta Cell Replacement
• immunosuppressive therapy	• insulin administration
• prevent beta cell exhaustion	• islet transplantation
• intracellular signal amplification	• mechanical artificial pancreas
• gene therapy	• bioartificial pancreas

Essentially, the biohybrid pancreas model is designed as a closed loop insulin delivery system meaning that insulin delivery is directly regulated by blood glucose level. This is accomplished by making use of the islets of Langerhans which are the body's natural regulators of insulin secretion. Under normal conditions, this regulation is fine-tuned by several humoral and neural factors which cannot be reproduced by a simple chemical or electromechanical system. For example, when a non-diabetic person consumes a meal, insulin secretion precedes the increase in blood glucose [Kraegen et al, 1990; 1981]. This anticipatory pattern, which has shown to establish glucose homeostasis, cannot be replicated by a man-made closed loop insulin delivery systems, but can be replicated by using immunoprotected islets.

Thus, a bioartificial pancreas which makes use of the natural regulators of insulin, the islets, seems to be the closest approach so far to the ideal of

sustained restoration of a self-regulating insulin supply. Although widely varying in designs, studies have shown that use of a biohybrid pancreas can effectively reduce glucose levels in diabetic patients. However, as will be discussed, there are certain design considerations and limitations that must be looked at further in order to achieve an ideal bioartificial pancreas.

7.2 Bioartificial Pancreas Designs

Several different bioartificial pancreatic systems have been researched and developed. However, almost all use pancreatic islets that are protected against immune rejection by a surrounding artificial membrane. There have been intravascular models in which the blood of the host circulates in contact with a membrane where the islets are placed on the other side in a closed compartment [Reach et al, 1981]. This is quite similar to a hemodialyser where the dialysate compartment has islets instead. There are also simpler extravascular systems such as microencapsulated islets, where individual or clusters of islets are surrounded by a membrane, or placed inside a hollow fiber configuration. These extravascular models have no vascular interface and are usually directly implanted inside the peritoneal cavity [Sparks et al, 1982].

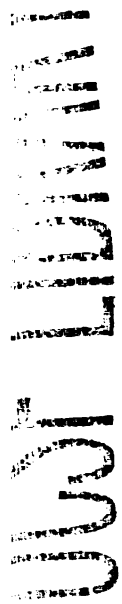
7.2.1 History of Bioartificial Pancreas

The development of the bioartificial pancreas began about four decades ago with studies of islet-containing perfusion devices implanted as arterio-venous shunts. The early devices were comprised of capillary fiber bundles seeded with isolated islets on their outside surfaces [Chick et al., 1975]. Using copolymers of polyacrylonitrile and polyvinyl chloride or

polytetrafluoroethylnene, these perfusion devices were usually fabricated through a phase inversion process. Membranes were structurally asymmetric, comprised of a dense retentive skin supporting a porous matrix within an acrylic housing. Since islets were suspended in a hydrogel matrix within an arterio-venous shunt, they benefited from exposure to higher oxygen tensions than extravascular devices. However, the use of these devices was largely unsuccessful due to rapid blood coagulation and clotting [Tze et al., 1976]. Subsequently, devices utilizing wider diameter tubular membranes were investigated. Several of these devices, using xenogeneic porcine islets, were shown to decrease insulin requirements for up to nine months in canines [Sullivan et al., 1991; Maki et al., 1993]. Nevertheless, several practical issues limited clinical success of such vascular perfusion devices. The size and geometry of the perfusion devices imposed a critical limitation on the amount of tissue that could be transplanted into diabetic patients. Moreover, the glycemic control of these perfusion devices was not optimal in terms of a rapid glucose-stimulated insulin secretory response.

Other configurations of hybrid artificial pancreatic devices took the form of tubular or planar diffusion chambers. These configurations provided the advantages of being extravascular and easier to retrieve, but had much lower oxygen tensions than intravascular devices [Maki, Ubhi, et al., 1991]. Various geometric configurations such as envelopes, straws, and spheres, usually placed in peritoneal or subcutaneous sites, have been studied. Algire and co-workers first proposed diffusion chambers for pancreatic replacement in 1949 [Algire

and Legallais, 1949]. In these devices, Millipore cellulose membranes were used to house transplanted tissue in order to study immune rejection. Although initial results were discouraging due to excessive fibrous overgrowth and cell death, this study paved the way for many other types of diffusion chambers . Permselective tubular membranes did achieve some success in rodent models [Lanza et al., 1991], however, few devices achieved therapeutic success in larger animals. Subsequently, Archer et al. (1980) studied islets placed inside Amicron XM-50 hollow fibers which were then placed in diabetic Chinese hamsters. Normoglycemia was achieved for several months but devices ultimately failed due to a fibrous tissue layer that developed around the implant. Likewise, Altman and colleagues (1988) investigated hollow fibers seeded with both allogenic and xenogeneic islets, but found that fibrotic reactions also limited their effectiveness. Lacy and coworkers (1991) fabricated acrylic copolymer hollow fibers that were biocompatible and reversed hyperglycemia in small animals, but was not as successful in larger animals. Some wider bore membranes with a smooth outer surface containing porcine, bovine, and canine islets were shown to restore normoglycemia in diabetic BB and STZ rats. One recent study showed insulin independence restored for more than 10 weeks in totally pancreatectomized dogs with canine-islet containing diffusion chambers [Lanza, Borland, et al., 1992]. Nonetheless, only allogeneic islets were transplanted, not xenogeneic. Moreover, laparotomies revealed extensive breakage of devices in vivo.



7.3 Microencapsulation

Due to the limitations associated with these larger device configurations, attention turned towards developing smaller bioartificial pancreatic devices, namely in capsular configurations. These capsules were designed to encapsulate individual islets or small clusters of islets to provide the advantages of greater surface to volume ratios and ease of implantation. However, this technology, utilizing various natural or synthetic polymers, also had several disadvantages which will be discussed next. As will be shown, the proposed microfabricated biocapsule is based upon such a capsular design and was developed to address some of the limitations associated with current polymeric microcapsule technology.

Encapsulation of biological substances, in essence, started with the creation of the first living cells by nature. It was only about forty years ago that humans began attempting to copy this natural process. Today, the technique of macro and microencapsulation is gaining acceptance and momentum as more scientists and engineers develop new applications and processes. Applications now range from such fields as graphic arts to agriculture to medicine. Of course, one of the more exciting prospects is encapsulation of living cells specifically for the treatment of diabetes.

Microencapsulation of biological materials within a semipermeable membrane was first reported by Chang in 1964 [Chang, 1964]. Microbial cells were enclosed within liquid membrane microcapsules. Gel entrapment and other methods of cell immobilization of microbial cells were also reported

[Vieth et al., 1979]. Early techniques of microencapsulation almost always involved cell-damaging conditions such as heating or using an organic solvent phase. The two most widely used methods of producing microcapsules, interfacial polymerization and phase separation, could not be used on live cells so new processes had to be developed.

Since then, research and development has proceeded at a rapid pace and has produced much excitement. Lim and colleagues refined the microencapsulation process for enclosing living cells [Lim and Sun, 1980]. These microcapsules remained morphologically and functionally intact for as long as 4 months in culture at 37°C and were able to achieve normoglycemia when implanted in rats for two weeks. The microcapsules were also small enough to be injected through an 18 gauge hypodermic needle. This microencapsulation technique involved the formation of microcapsules with a new membrane system made of polyelectrolyte hydrogel complexes, specifically alginate polylysine. The ability of alginates to form gels with divalent and multivalent ions such as calcium served as the basis for microencapsulation procedures. Alginates were used to form semipermeable hydrogel encapsulation membranes by co-reacting them with polycations such as polylysine.

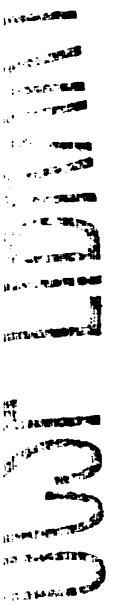
Contrary to previous processes, this encapsulation method involved no cell-damaging conditions. Essentially, this microencapsulation process involved suspending living cells in sodium alginate solution. The cell suspension was then extruded through a microdroplet forming device,

producing microdroplets which fall into a $CaCl_2$ solution to form gelled microbeads with the cells entrapped. These cell enclosing microbeads are then treated with a solution of polylysine which displaces the surface layer of calcium ions and forms a permanent polysalt membrane. The outer edge of the gel structure is converted to alginate polylysine complex to form a membrane. Then the interior calcium alginate gel is liquefied either to stay in or to come out of the capsule with a calcium sequestrant such as buffered sodium citrate solution [Lim, 1984].

In the polylysine alginate capsule fabrication process, the initial step of temporary micro gel bead formation by a divalent ion (Ca++) controls the membrane formation in terms of thickness and pore size. This initial research on alginate capsules started the upsurge of investigation into different encapsulation materials using various polyelectrolyte solutions cross linked with polyelectrolytes of opposite charge, as well as alternative polymeric systems for cell encapsulation.

8. General Biocapsule Design Requirements

Researchers have determined that a continuous and self-regulating supply of insulin is the most critical requirement of an artificial pancreas. A hybrid pancreas that makes use of isolated pancreatic islets is probably the best approach to a self-regulating insulin supply and possibly the only approach to a safe, low risk treatment. It is a method of treatment that minimizes the amount of foreign components and materials introduced into the body. In contrast to conventional insulin therapy or mechanical artificial pancreas models, a bioartificial pancreas makes use of the body's natural functions to get the most ideal blood sugars.



Regardless of the type of bioartificial pancreas configuration chosen, all immunoisolation biohybrid devices must meet certain common design criteria. It is possible to design both intravascular and extravascular bioartificial pancreases, but either model must be able to respond *rapidly* to changes in blood glucose levels. Ideally, by having pancreatic islets surrounded in some fashion by a membrane, they are protected against immune rejection and can, therefore, survive for long periods of time, given sufficient nutrients. To be effective, the hybrid pancreatic system should satisfy several basic functions.

Overall, the system should perform as a closed loop delivery system that responds to a glucose load by an increase in insulin production in order to treat diabetes in a physiological manner. For this to occur, the biocapsule must be fabricated taking into account several essential design criteria. First, in additional to mechanical and chemical stability, the entire device must be

biocompatible to both the host tissue surrounding the implant and the encapsulated cells. The issues of biocompatibility must be considered in all fabrication protocols and post-processing steps. For living cells and tissues to stay viable and undamaged during and after encapsulation, the membrane must be completely non toxic to cells and tissues as well as non-immunogenic and non-thrombogenic. Furthermore, the cells inside the biocapsule must retain their *viability* and *functionality long-term* in an immunoprotected state. This can only be achieved by designing a biocapsule membrane with adequate *transport properties* whereby essential nutrients and metabolites such as insulin, glucose, and oxygen can diffuse freely in and out of the capsule. Central to the technology is the fact that the membrane must provide effective *immunoprotection* for the encapsulated cells.

8.1 Fabrication Issues: Processing, Stability, and Scaling

The ultimate success of a given biocapsule depends critically on how the capsules are fabricated and how long they can maintain stability both outside and inside the body. As mentioned previously, focus has been placed entirely upon polymeric biocapsules. However, the preparation of defect-free polymeric capsules has not been easy and has led to numerous problems associated with instability and biodegradation. This is just one reasons why alternatives to polymeric capsules are being sought. The quality of the capsule can vary greatly depending on its chemical and physical properties.

Capsule strength plays an important role, especially in vivo. When the capsules are not strong enough, they start to collapse within a few days after

transplantation [Fan et al., 1990]. Even though membranes for cell encapsulation rarely have to withstand pressures over 10 PSI (500 mmHg) or temperatures above 37°C, they are still subject to rupture and degradation within the body. Under environmental and chemical stresses, imperfections in a polymeric capsule wall will decrease its durability leading eventually to capsule rupture. In addition, the membrane must be strong enough to withstand repeated mechanical motions such as shaking or stirring inside culture flasks and surgical manipulation and implantation [Lim, 1984].

Capsules made from polymers are extremely susceptible to chemical stresses. Because polymers often have reactive organic side groups, they can undergo hydrolysis or esterification and are not always reliably stable in the body's chemically active environment. Furthermore, obtaining the proper sphericity of the capsules or the appropriate surface smoothness is quite important to the overall in vivo duration of the capsules. For polymer capsules, solute concentration is one of the parameters which can significantly affect capsule strength by affecting capsule formation. For example, studies have shown that a small decrease of the alginate concentration from 1.2 to 1.1 percent (w/v) results in a dramatic reduction of the fraction of perfectly spherical and smooth microcapsules from 100 to 25 percent [Chen et al., 1992].

The polymer-crosslinking agent contact time is another significant factor which affects the strength of the capsule membrane [Sun, 1988]. For instance, an alginate polylysine reaction time above six minutes reduces the flexibility and durability of the capsules. This may be due to an increase in capsule

membrane thickness or wall density with reaction time that makes the capsule less flexible.

In terms of practical issues having to do with the implantability of the artificial pancreas, there is a dearth of studies on the adaptation of the bioartificial pancreas to large animals such as humans. It was demonstrated that a vascular device containing 50,000 islets inside the outer department and perfused with a synthetic medium produced almost 20 units of insulin per day[Reach, 1990]. This is about one third the production of insulin by a healthy adult human pancreas. If one considers an extravascular system made of hollow fibers, 300,000 islets constitute a packed volume of 1 ml. This volume would require a 130 cm long fiber with a 1 mm diameter. Questions are thus raised as to whether the body can support certain islet package volumes and whether these package structures can support islet function and oxygenation.

Microencapsulated islets carry similar problems associated with the volume of the implanted islets. Based on average volumes found in several publications, the volume of implanted islets in humans needs to be over 60 ml to be effective in reduction of glucose levels. The implantation of such large volumes of foreign material inside the peritoneal cavity can cause serious biocompatibility problems such as fibrosis and other inflammatory reactions. Thus, these issues of scaling indicate that the application of a bioartificial pancreas in humans is still far off.

8.2 Biocompatibility

The biocapsule, like most biomaterials, must be sterile, non-pyrogenic, non-toxic, non-complement activating, and non-hemolytic. Additionally, capsule materials should be free of leachables or extractables, especially when in contact with blood. Blood clotting is a serious problem associated with intravascular implants. The prevention of blood clots anywhere within the device or at the connection to the blood vessels must be achieved without chronic systemic anticoagulation. While the development of non thrombogenic surfaces is difficult, it is much needed. Aspects of hemocompatibility should be kept in mind when designing the geometry of the system. Decreasing the resistance to flow has proven to be a good way to decrease the thrombogenic risk [Colton and Avgoustiniatos, 1991]. As fluid mechanics demonstrates, the less kinks or imperfections in blood channels, the less likely is it that thrombi will form. Another possible solution is the use of heparinized surfaces or heparin-like biomaterials. Heparin is an anti-coagulant that will prevent aggregation of platelets thus reducing the thrombi formation.

Likewise, for the extravascular system, we see biocompatibility problems. Many of the implants are associated with fibrosis, a foreign body reaction to implanted materials. Fibrosis is a complex process characterized by: acute inflammatory response, chronic inflammatory response, and development of granulation tissue that ultimately leads to the fibrotic tissue capsule that is composed largely of collagen, macrophages, fibroblasts, and few if any capillaries[Anderson, 1988].

An important determination of biocompatibility is the interaction of macrophages with the surface of the material of the implant. Foreign body response is influenced by surface chemical composition, implant shape, morphology, and detailed microscopic topography at the material-tissue interface. The less pure and smooth the implant is, the more likely is a inflammatory reaction to occur. Inflammatory reactions around the membrane have been described with the hollow fiber configuration and with microcapsules[Moussy et al., 1988].

Furthermore, it was discovered that certain concentrations and mixtures of polymers were not biocompatible. Polylysine was found to be not as biocompatible as alginate and tended to promote the attachment of fibroblasts. Due to the fact that no biomaterial is 100% biocompatible, biocapsules will, for the most part, eventually attract cell overgrowth after several days to weeks. Factors such as sphericity, smoothness of the capsule surface, and the composition and sterility of the polymer play an important role in preventing overgrowth. In addition, sometimes imperfections that occur in the microcapsular membrane can permit leukocyte infiltration and graft rejection of the implanted capsules[Wong and Chang, 1991].

It is often noticed that early graft failure of microencapsulated islet grafts is accompanied by a marked foreign body type reaction and islet damage [Cole et al., 1992]. Some recovered polymeric microcapsules are covered with a tissue reaction in which giant cells, macrophages, and fibroblasts predominated but also include other mononuclear cells. Although microcapsules are

impermeable to cytotoxic antibodies, smaller molecules such as cytokines can sometimes cross the microcapsule membrane in vivo. They may be responsible for the islet damage and graft failure observed within intact capsules. In addition, the reactive cells create a physical barrier to the passage of islet nutrients and also consume the nutrients that was meant for the islet. This leads to the starvation of islet cells and consequently rapid decline in function.

A major goal with the bioartificial pancreas is extensive vascularization at the tissue membrane interface in order to minimize mass transfer resistances and diffusion time lags associated with the tissue. This can be achieved by a more thorough understanding of angiogenesis, the formation of new blood vessels. There is a lack of knowledge concerning the promotion of angiogenesis by synthetic materials and structures. Currently investigations are underway to understand these reactions and develop a truly biocompatible material interface.

8.3 Preserving Islet Viability and Functionality

It is realized that encapsulation of the islets of Langerhans in a protective biocompatible membrane is a potentially effective way to prevent graft rejection in allotransplantation or xenotransplantation without the need for immunosuppression. The semi-permeable membrane which surrounds each islet should allow such molecules as peptides and nutrients to flow freely while barring the passage of larger molecules such as immunoglobulins, thus

eliminating the need for immunosuppression therapy. Thus, the process of encapsulation may be able to overcome the limitations of islet transplantation.

Human pancreatic islets, as discussed earlier, have a diameter of about 150μm and about 60 percent of the islet mass are beta cells that secrete insulin. There are about 1 million islets in a human pancreas. However, only about 10 percent should be enough to provide normoglycemia in a 70 kg patient [Galleti, 1993]. Islet transplants have been shown to prevent complications associated with diabetes in experimental animals.

It is critical that the secretion of insulin and glucagon by microencapsulated islets of Langerhans be qualitatively similar to that of unencapsulated free islets[Levesque et al., 1992]. However, studies have shown that the quantitative response of islets microencapsulated in polymeric membranes is often significantly reduced for insulin. A possible explanation for this decreased secretion of insulin is that the polymeric microencapsulation process induces a selective loss of insulin from the islets. Analysis of the hormone content of islets immediately after encapsulation revealed significant reductions in both insulin and glucagon content. This probably occurs due to damage to the islets or significant stimulation of hormone secretion by one of the reactant solutions utilized to create the membrane capsule.

For polymeric capsules, the size is often heterogeneous and hard to control, ranging from 300 to 800 micrometers. However, for successful encapsulation of islets, it has been observed that controlling the size of the capsules is critical. The membrane surface area of an islet compartment has to

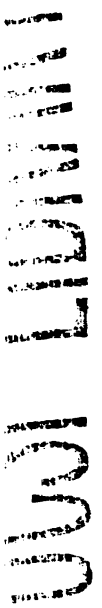
be large enough to support the cells which must spread evenly and function properly. If membrane surface area is not large enough, it can cause anoxic or hypoxic conditions for the islets. Studies demonstrate that islet insulin secretion is detrimentally affected by hypoxic environments at pO_2 levels that are substantially higher than those required to compromise cell viability. Careful attention must be paid to device size in order to minimize the effect of oxygen diffusion gradients.

However, since the volume of the islet chamber is involved in the response time of the system, it is important the capsule not be too large. It has been demonstrated that size has a major effect on the performance of microcapsules [Chicheportiche and Reach, 1988]. The data clearly demonstrates that small polymeric capsules (mean diameter, 350 mm) containing isolated islets of Langerhans respond rapidly (within 15 minutes) to stimulation by increasing insulin secretion and that this is not the case for large microcapsules (650 mm) from the same batch where the size of the islets within the two kinds of capsules was similar.

This phenomenon may be explained by two hypotheses. First, possibilities for exchange across the microcapsule membrane decrease as the capsule diameter increases, since they are related to the surface over volume ratio. So, the absence of response of islets enclosed in large capsules might be due to an inadequate influx of factor essential to islet survival or function or to the accumulation inside the capsule of an inhibiting substance. Secondly, islets contained in small and large capsules secrete the same amount of insulin in

response to glucose. Thus, intracapsular insulin concentration will be much smaller in large microcapsules. Since the driving force for insulin diffusion is the difference in insulin concentration across the membrane, this can explain a slower insulin release from the large microcapsules [Chicheportiche and Reach, 1988].

The formation of microcapsules of reduced size allows for many other improvements according to a study done by Lum et al. (1991). Smaller capsules allow for increased cell viability, because encapsulated cells have easier access to oxygen and nutrients. There is faster cell response to glucose fluctuations because dead space is reduced. In addition, there is greater mobility of the cells, thus lessening the mechanical damage to the capsules when implanted. All of these reasons show that the volume of the microcapsule should be considered for the optimization of the microencapsulation procedure.



Finally, the overall safety of the bioartificial system has to do with the quality of the isolated islets, the sterility, and the absence of pyrogens in the implanted materials. To ensure safety and efficacy of foreign cells, they must be characterized for functional ability, stability in a sequestered environment over several cycles of replication, and virus-free status before and after incorporation in the device [Galleti, 1992]. In addition, the membrane production process also has strict quality control requirement. Focus should be placed on issues of retrievability, membrane decay in a dynamic environment such as the body, and any consequences that might arise due to escape of cells

from their membrane envelope. The issue of safety should be looked at with circumspection. Care has to be taken in estimating the risk-benefit ratio, since insulin-dependent diabetes mellitus can be treated, with limitations, using insulin injections.

8.4 Immune Mechanisms and Immunoprotective Properties

Upon implantation of foreign cells, an immunogenic response immediately develops. This sequence of events begins with the binding of antibodies (either IgG or IgM) to both cell surface and soluble antigens. Subsequently, complement activation is initiated and the complement cascade can form a membrane attach complex (MAC) that leads to cell death. The molecular weight of IgG, the smaller antibody, is approximately 160 kD with dimensions between 15 and 40 nm [Colton, 1995]. Its diffusion is often regarded as the rate determining step of islet cell destruction.

It has been suggested that a membrane that prevents the passage of a 30 nm particle will retain both C1q and IgM, but not necessarily the smaller IgG [Colton, 1995; Colton and Avgoustiniatos, 1991]. Both IgM and C1q are larger than IgG, so if passage of host IgG across the immunoisolation barrier can be prevented, then specific antibody mediated destruction of islet cells should be also prevented. Previously, it had been assumed that having a membrane with a nominal molecular weight cutoff of 50,000-100,000 Daltons would be sufficient. This is now known not to be the case. The situation is much more complicated due to the size discrimination of most membranes.

Most often, with polymeric membranes, transport is associated with the membranes ability to retain marker molecules. This is described in terms of a nominal molecular weight cutoff. Although these measurements give an approximation of what size biomolecule can pass through the membrane, they do not describe the transport rate or selectivity of the membranes. In order to develop the optimum membrane, a fundamental understanding of the humoral immune rejection mechanism must be achieved, as well as the absolute dimensions of the immune markers of interest. Only then, will we know which molecules can be allowed to pass through the membrane and which are not. Current knowledge in immunology is far from providing this information.

8.5 Membrane transport and kinetics

In applications ranging from biotechnology to water purification, the development of suitable membranes is a much sought after goal. Over the past thirty years, the field of membrane science and technology has developed rapidly. Interestingly enough, the human body employs several types of membranes for many of its important physiological processes such as separation, concentration, purification, and isolation.

There are several special features that a membrane for a biohybrid pancreas must possess. Achieving satisfactory glucose-insulin kinetics is a major obstacle to the development of such a hybrid pancreas[Reach et al, 1984]. From a mathematic standpoint, it was concluded that minimizing the volume of the islet compartment was of utmost importance in reducing the lag in insulin release. However, this solution is limited by the size of the islets and

by an inhibitory effect of insulin on islet cell function that has been shown to be enhanced by reduced islet chamber volume.

The size of the cell compartment or microcapsule is extremely important because it significantly affects microsolute and macrosolute transport. One must worry about the volume of the system since kinetics of the response might be too slow if the volume of the islet compartment is too large. Large diffusion distances can cause a decrease in insulin secretory response. It is generally accepted that the response time has to be shorter than 15 minutes to avoid overexcursion of blood glucose and hyperinsulinemia after a meal[Kraegen et al., 1981].

Achieving the desired functional membrane properties of permeability and permselectivity are of utmost importance. Understanding the transport characteristics of immunoisolation membranes is central in evaluating overall performance and functionality of biocapsules for cell transplantation. Although membranes can be fabricated in a multitude of ways using a variety of materials, basic transport characteristics must guide membrane selection criteria. The membrane, as mentioned earlier, must restrict the passage of larger immunomolecules and shed antigens, while permitting free passage of small molecules required for islet metabolism and hormonal release.

Almost all immunoisolating micro- or macrocapsules are diffusion based. Transmembrane diffusion must take into account both membrane and bath components, as well as their boundary layers. Diffusion of antibodies

such as IgG through the biocapsule membrane can be described by Fick's second law,

$$\frac{\partial C}{\partial t} = D\left(\frac{1}{r^2}\right)\frac{\partial}{\partial r}\left(\frac{r^2 \partial C}{\partial r}\right)$$

where D is the diffusion coefficient for IgG through the biocapsule membrane, r is the distance, t is the time, and C is the concentration of IgG at distance r.

For most solutes of interest, their exact size and conformation are unknown. Therefore, an equivalent spherical solute radius is usually specified. In general, the diffusion of molecules in solutions relative to a membrane can be described by the Stokes-Einstein equation, $D=RT/6\pi\eta\sigma N$. This equation is useful for analyzing solute flow through pores in membranes, if the pores are large compared to the solute.

In a recent study by Dionne et al. (1996), the diffusion of low and high molecular weight marker solutions of glucose and IgG, respectively, through hollow fiber membranes were examined. They found that the passage of glucose through test membranes was reduced by a factor of 50% compared to what it would be in water and the passage of IgG was reduced by 4 orders of magnitude. The study concluded that anisotropic ultrafiltration membranes permitted the passage of small amounts of high molecular weight species despite membrane nominal molecular weight cut off parameters and were still capable of xenogeneic immunoisolation.

Similarly , Burczak et al. (1994) looked at the passage of IgG, insulin, and glucose across PVA hydrogel membranes. It was also found that IgG

permeability was not completely prevented through prepared PVA membranes. It was hypothesized that since the IgG molecule was not spherical, even a mesh size as small as 90 Å might not completely block its passage. Moreover, because of the distribution of the mesh size across the membrane, it was not possible to find the exact cut off dimension for IgG. Nonetheless, it was concluded that the PVA hydrogel membranes were sufficient for immunoisolation because of their ability to greatly restrain IgG diffusion, even while not completely blocking it.

For traditional polymer capsules, final evaluation of the surface finish, wall thickness, and membrane uniformity is often done by scanning electron microscopy. The optimum results would show a smooth interior and exterior surface with membrane thickness of about 4 to 5 μm. However, since the polymeric microcapsular membrane is formed by the layering and criss-crossing of linked polyelectrolyte complexes, the diffusion paths through the membrane pores are uneven instead of linear tunnel-like holes. The issue of porosity is important to consider in order to ensure the prevention of immune rejection. The design of the membrane must take into account the largest molecule that it can be permeable to without endangering the tissue. It is difficult to precisely control considering the tortuosity and broad pore size distribution present in most polymer capsules.

In polymer capsules, the permeability of the capsule membrane depends on the viscosity average molecular weight Mv and the average molecular weight M of the polylysine used in the encapsulation process. The lower the

Mv, the less permeable the capsules. Membranes made with M > 40 kD allowed for the diffusion of proteins such as serum albumin and hemoglobin and are, therefore, unsafe for the protection of pancreatic islets against humoral factors of the immune system. However, when membranes with cutoffs of M= 17 kD or 25 kD were used, diffusion of these proteins was completely stopped [Sun, 1988].

8.6 Effectiveness of Polymer Capsules

While scientists have come close to developing a bioartificial pancreas that satisfies many of the design requirements, there are still several problems associated with implantability and effectiveness that are crucial to future success and have yet to be resolved. Although microcapsules using several different polymers have been investigated, most of the work has been done on the alginate polylysine system. Some of the other materials used include chitosan-alginate, polyacrylate, PAN-PVC, and agarose. These polymeric systems have met with four main problems associated with: 1) lack of control of microcapsule morphology and permeability; 2) insufficient mechanical and chemical stability in vivo; 3) inadequate biocompatibility in terms of fibrotic encapsulation; and 4) insufficient molecular cut-offs due to non-uniform membrane pore sizes.

A few studies have demonstrated that capsules made of the alginate polylysine membrane survived in situ for nearly 1 year after transplantation [Leung et al, 1983; O'Shea et al., 1984] indicating that the membrane was

biocompatible. Using the encapsulation method described previously, greater than 90% of the islet cells retain their viability and functional properties. But, other studies showed alginate capsules were not biocompatible and that a fibrous tissue capsule formed around the alginate microcapsules when implanted in vivo [Waterfall et al, 1991; Soon-Shiong et al., 1991; Chicheportiche et al., 1991]. These discrepancies on biocompatibility were explained in terms of the concentration and purity of the polymer solutions used to make the microcapsules.

When rat islets were encapsulated and transplanted into diabetic rats and mice, the diabetic state was reversed[Fan et al., 1990; Lum et al., 1992]. However, in most of the studies, diabetes was induced by the injection of streptozotocin. There was a short term (1-3 months) 20-30% reduction in insulin requirement Several laboratories reported that microencapsulated islets were rapidly destroyed when implanted into either NOD mice or BB rats, animal models of spontaneous autoimmune diabetes. Even allografts in spontaneously diabetic mice resulted in only a short period of normoglycemia [Calafiore et al, 1990]. One study did suggest prolonged function of rat islets xenografted into NOD mice using improved microcapsules [Lum et al., 1991]. However, more often than not, investigators found intense cellular and fibrotic reactions to polymer-microencapsulated xenografts. Furthermore, although micro-encapsulated canine islet allografts in spontaneous diabetic dogs were shown to be successful long term [Soon-Shiong et al., 1992], xenografts implanted in larger animals have not met with the same success.

There are still questions as to the absolute requirements of a semipermeable membrane to ensure that immune rejection is prevented. Despite its obvious importance to the central concept of immunoisolation in a bioartificial pancreas, this issue has received little attention. The key in designing a membrane is knowing the largest pore size that can be present or the smallest molecule whose passage must be prevented without increasing the risk of immune rejection. Polymeric membrane permeability is usually controlled by flow velocity, viscosity, and temperature conditions during fabrication. However, such processes seldom result in uniform membranes.

Initial enthusiasm for microencapsulation technology for the immunoisolation of xenogeneic islets has been lessened due to the unresolved technical issues discussed above that have yet to be sufficiently addressed and overcome. Several studies have shown that microencapsulated islets, with a variety of encapsulation materials and methods, are still rapidly destroyed by recipients with spontaneous autoimmune diabetes such as NOD mice [Ricker et al., Darquy et al., Weber et al., Mazaheri et al., Calafiore et al.]. Although there have been a few cases of prolonged islet survival and function of rat islets xenografted into smaller animals, the majority of investigations have been unsuccessful in maintaining cellular function. Being able to perhaps address and overcome some of these challenges in encapsulation technology is the prime motivating factor for research and development of the microfabricated biocapsule.

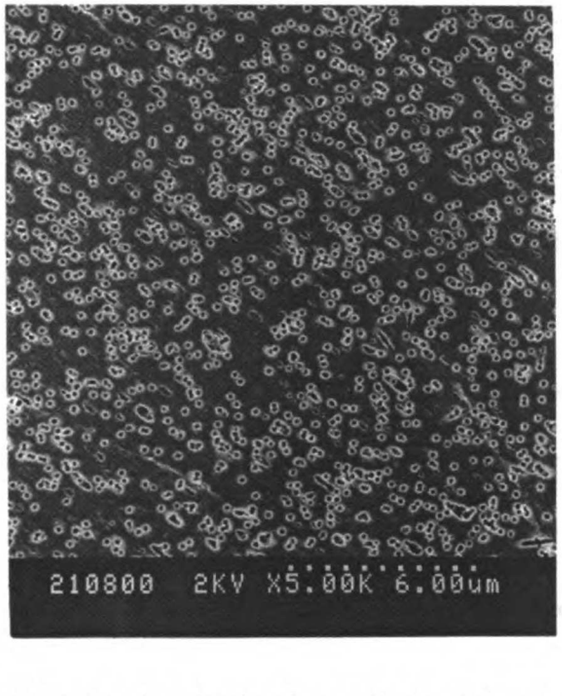


Figure 8.6.1 Commercial Tracked Etched membrane Nominally 0.2 micron poresize (Nominal porosity 8%) [Chen V et al., 1997]

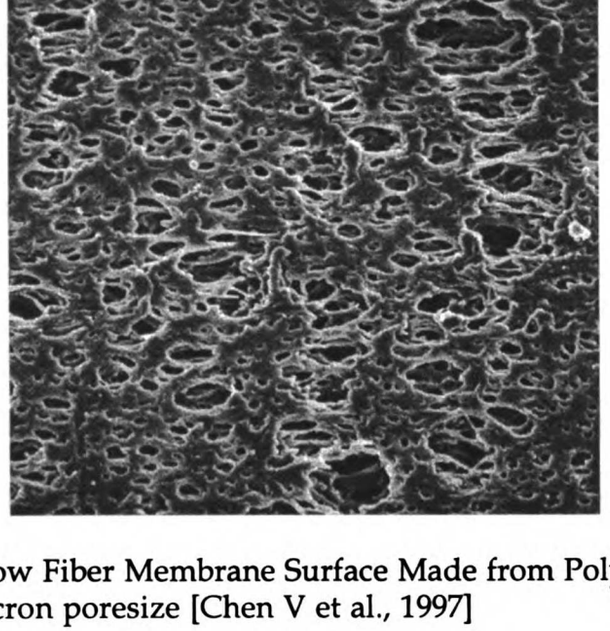


Figure 8.6.2 Hollow Fiber Membrane Surface Made from Polyethersulfone. Nominally 0.2 micron poresize [Chen V et al., 1997]

Although the development of a bioartificial pancreas seems to be a promising method of therapy for diabetes, there are still many limitations due to the problems of instability, biocompatibility, and immunoprotection that are associated with the production of long term functional designs. It was found that survival rates of encapsulated cells in polymeric membranes was strongly affected by 1) mechanical strength of the capsules, 2) non-clustering and agglutination of the biocapsules; and 3) appropriate diffusion rates. For these reasons, there is a need to investigate new biocompatible materials and employ innovative strategies to achieve immunoprotection for improvements in biocapsule technology. As will be made clear in the next section, the microfabricated biocapsule possesses mechanical strength due to the inherent mechancial properties of silicon and the use of membrane reinforcing ribs. Section 10 describes the biocompatibility and chemical stability of the proposed biocapsules. This property, along with the use of different surface coatings, will allow the capsules to remain stable in vivo and not degrade or agglutinate over long periods of time, in contrast to most polymeric capsules. And most importantly, the geometrical configuration and membrane diffusion rates of the microfabricated biocapsule can be precisely manufactured and are critical for cell viability and immunoisolation, as discussed in sections 11 and 12. This will allow for improvements over polymer-based biocapsules in terms of pore sizes and distributions necessary to achieve immunoprotection. Finally, in chapter 13, the feasibility of an functional implantable microfabricated immunoisolating biocapsule is made apparent through *in vivo* studies in small

animals. The lessons learned by previous researchers as well as the advances in material science and fabrication technology have proven useful in the realization of an unprecendented microfabricated biocapsule that may potentially alleviate many of the problems associated with conventional polymeric biocapsules.

9. Biocapsule Design and Fabrication

A crucial advantage of microfabrication technology is the ability to fabricate membranes of specific pore size, allowing one to optimize the biocapsules specifically for the encapsulation of pancreatic islets. Current polymeric biocapsules have not been able to achieve uniform pore size membranes in the tens of nanometer range. By contrast, several variants of microfabricated diffusion barriers have been created, containing pores with uniform dimensions as small as 20 nm [Ferrari et al, 1996]. Furthermore, improved dynamic response of islets tissue can be obtained due to the reduced membrane thickness (9 μm) of microfabricated membranes compared to polymeric membranes (100-200 μm). It is important to retain rapid intrinsic secretion kinetics, in particular first phase insulin release [Colton and Avgoustiniatos, 1991], so as to provide physiological feedback control of blood glucose concentrations.

Microfabricated biocapsules with membrane pores in the tens of nanometer range seem suitable for application in xenotransplantation. The typical dimension of insulin, glucose, oxygen and carbon dioxide, molecules which should pass freely through the membrane, is less than 35 Angstroms. The blockage of immune molecules, however, is a much more complicated task. As discussed earlier, the immunoisolation membrane should prevent the passage of either host C1q or IgG to remain effective, suggesting that an effective immunoisolation membrane should have maximum pore diameters of 30 to 50 nm [Colton, 1995].

Nonetheless, it is not a simple manner to select an appropriate cut off dimension for the immunoisolation membrane to create a suitable biocapsule. Permeability of glucose, insulin, and other metabolically active products, must be high enough to insure therapeutic effectiveness. All previous immunoisolation membranes, due to their polymeric nature, have found that meeting these cut-off requirements is quite difficult, due to the broad pore size distribution of real membranes. Even if only 1% of pores are larger than the cut-off goal, the pores would allow the passage of antibodies in sufficient amounts to initiate immunorejection pathways.

The microfabricated silicon-based biological containment provides an alternative solution to the problem of cell xenograft protection by applying fabrication techniques developed originally for MEMS to realize an immunoisolating biocapsule. The first challenge was to apply the technology of microfabrication to a bioartificial system capable of supporting physiological processes. Although contemporary advances in processing now permit the fabrication of sub-millimeter silicon-based devices with features in the tens of nanometer range, it was still a major undertaking to develop ways in which this technology could be effectively applied to a implantable biocapsule. The first embodiments of the biocapsule used silicon as its primary material not only because of silicon's availability and processing compatibility but also due to its unique properties. It was thought that the silicon biocapsules could provide the added advantages of mechanical stability, uniform pore size distribution, and chemical inertness. By taking advantage of silicon bulk and

surface material properties, structures can be engineered to perform specific functions. Thus, microfabrication technology may be advantageous in the field of tissue engineering by creating precisely controlled microenvironments to stimulate and enhance transplanted cell behavior.

As mentioned earlier, because of the numerous design considerations and constraints of a biological environment, the prototypical biocapsule was not realized overnight. In fact, there were several design problems to be considered and overcome. The evolution of the present biocapsule design and device fabrication took several years. The fabrication began with the creation of several variants of microfabricated diffusion barriers, containing pores with uniform dimensions ranging from 1 micron down to as small as 18 nm [Chu et al., 1995; Ferrari et al, 1996]. It was not entirely clear until after in vitro testing which pores dimensions would be suitable for a biocapsule, even through it was hypothesized that pores should be in the 15 to 100 nm range. Eventually other design consideration aside form pore size were examined such as membrane thickness and pore path length and configuration.

Once the basic technology for the membrane portion of the biocapsule was developed and established, it was yet another challenge to integrate this technology into a capsular configuration capable of housing and maintaining biological cells. In the following sections, step by step details of several different biocapsule fabrication protocols are presented, and their advantages and disadvantages are discussed.

9.1 Fabrication Concepts and Techniques

The silicon biocapsule, as a whole, is comprised of two main parts. The first and most critical component is the filtering membrane which governs nutrient diffusion and cell immunoisolation. This membrane area is made of thin layers of polysilicon, silicon dioxide, and/or single crystalline silicon depending on the design employed. The other main part of the biocapsule is the anisotropically backside etched wafer which forms the biocapsule well in which cells were placed.

Since photolithography, in general, is not amenable to the fabrication of pores with dimensions smaller than 0.25-1 micrometer, strategies using sacrificial layers were employed to achieve desired pore sizes down to the tens of nanometer range. The strategies developed were initially based on the use of a sacrificial oxide layer, sandwiched between two structural layers, for the definition of the pore pathways [Chu and Ferrari, 1995]. This strategy encompassed a multitude of viable embodiments for the biocapsules. However, all designs of the microfabricated biocapsule consisted of a surface-micromachined membrane on top of an anisotropically-etched silicon wafer, which provides mechanical support [Ferrari et al., 1995].

The biocapsule membrane was fabricated using several different protocols, which allowed membranes to be optimized for biological encapsulation studies. In addition, the specific configuration of the membrane pores, including pore size, density, and path length, was fabricated as needed, depending on encapsulated cell type and desired diffusion kinetics.

9.2 *Culture Wafer Fabrication*

To begin studying the biocapsule properties, two different culture wafer (otherwise referred to as half biocapsules) were designed and tested. As will be shown, both designs utilized the composite sandwiched membrane structure to achieve the desired pore size, density, and configuration. Two types of silicon cell culture wafers were designed and fabricated for biological studies. Both cell culture wafer designs consisted of the surface-micromachined membrane on top of an anisotropically-etched silicon wafer. The major differences between the two types of cell culture wafers were their basic geometries and pore sizes. As can be seen in Figure 9.2.3 and Figure 9.3.1, both biocapsule designs had wells with semi-permeable membranes, which contained the cells. Depending on the desired pore size, this membrane area could be made from only one p^+ silicon layer or sandwiched p^+ silicon/ silicon dioxide/p^+ polysilicon layers, as will be described in detail later.

The typical thickness of the p^+ silicon membrane in Design I was 6μm. In Design II, the total thickness of sandwiched membrane structure was about 9 μm. The cell culture well had total dimensions of 2 mm x 2 mm on the non-membrane side with a well depth of 550 μm. The volume that a single well can hold is about 1.3 ml. The typical membrane area in both designs is around 1.5 mm^2. The pore size in Design I, 3μm by 3μm, is determined solely by photolithography. The pore size in Design II , 78 nm, however, is determined by a deposited thin oxide sacrificial layer. Figure 9.2.3 shows a micrograph of the cell culture well membrane area of design II. In the membrane area of

Design I, the area ratio of 3 x 3 μm pores to the solid p^+ silicon is 5.5%. In Design II, the area ratio of pore channel to solid area is about 1.23×10^{-5}.

Fabrication for both culture wafer design begins with p-type, 20-50 W-cm, 4" (100) silicon wafers. In Design I, the silicon membrane is made of heavily boron doped silicon (p^+ silicon). The diffusion channels are generated by direct etching through the p^+ silicon film using a plasma etcher. The etched holes on the p^+ silicon will be the diffusion channels for the cell culture wells. The resolution of the photolithography determines how small and how dense the pores can be on the cell culture wafer membrane.

To fabricate Design I cell culture wafers, the wafer is first cleaned and heavily boron doped using a boron solid source at 1125°C for 6 hours. The wafers are then wet oxidized at 950°C for 30 minutes in order to ease the removal of the borosilicate glass formed during the p^+ diffusion. The wafers are then stripped of their surface borosilicate and the surface sheet resistance is measured for the doping uniformity. The measured surface sheet resistance is 1.24+/- 0.04 W/square. The estimated etch-stop concentration ($7 \times 10^{19} cm^{-3}$) is around 6μm below the silicon surface. The device wafer is wet oxidized again to achieve a 0.38 mm silicon dioxide layer for later p^+ silicon etching.

For Design I, arrays of 3 μm by 3 μm holes are photolithographically defined and etched into the p^+ silicon using a Lam Research Rainbow plasma etcher. The etch recipe parameters are Helium flow rate : 400 sccm; Cl2: 180 sccm; Base pressure: 425 mTorr; RF power: 275 W; Etch rate: 0.3-0.5 mm/min. The oxide is then removed using buffered oxide etch. A 2 μm low pressure

chemical vapor deposition phosphosilicate glass (LPCVD-PSG) is deposited on both sides of the device wafers and further annealed at 1000°C. Subsequently, 2 mm by 2 mm etch holes are patterned on the backside of the surface oxide. Device wafers are etched in ethylenediamine-pyrocatechol (EDP) at 100 mC for

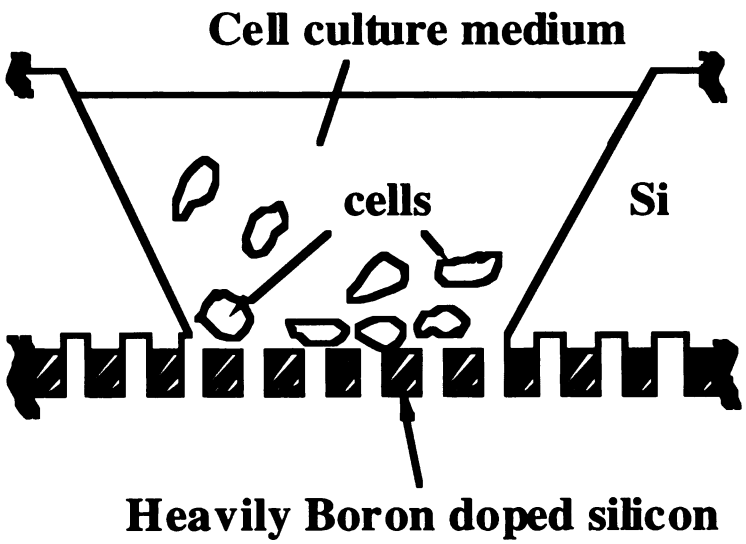


Figure 9.2.1 A schematic diagram of a cross-sectional area of an unit of the Design I cell culture wafer (not to scale). The 3 by 3 μm pores are determined solely by photolithography.

about 10 hours. The silicon etch rate in EDP decreases sharply when the boron concentration inside the silicon is larger than $7X10^{19}cm^{-3}$. This leads to a p^+ single crystal silicon membrane after 10 hours with well defined pore sizes. The etched wafers are rinsed in deionized water and 5:1 buffered oxide etch for 30 minutes to release the flow channels. Finally, the released cell culture wafers are further cleaned in Piranha solution (1:1 sulfuric acid: hydrogen peroxide) for 10 minutes and rinsed completely with deionized water.

In Design II, the diffusion membrane is made of a layer of p^+ single crystal silicon, p^+ polysilicon and a sacrificial thin silicon dioxide layer. Unlike in Design I, photolithography alone *does not* determine the flow channel pore size. As can be seen in Figure 9.2.3, the filtration channels in this design are generated by releasing the sandwiched silicon dioxide layer.

The processing of Design II wafers begin with the same p^+ diffusion and silicon plasma etching of 2μm x 2μm arrays as described earlier for Design I (Figures 9.2.2 and 9.2.4). A 53 minute 850°C low temperature dry oxidation is used to produce the 78 nm sacrificial silicon dioxide used for the cell culture wafers. This oxide layer is the channel oxide that controls the pore size and is removed by BHF during the last stage of the fabrication. Because of the well-controlled oxidation environment, the variation of the pore channel oxide layer is less than 10% over a 4" wafer. By changing the oxidation time, temperature, and gas composition, silicon membrane pores ranging from several tens of

nanometers to several micrometers can be fabricated easily and with great accuracy.

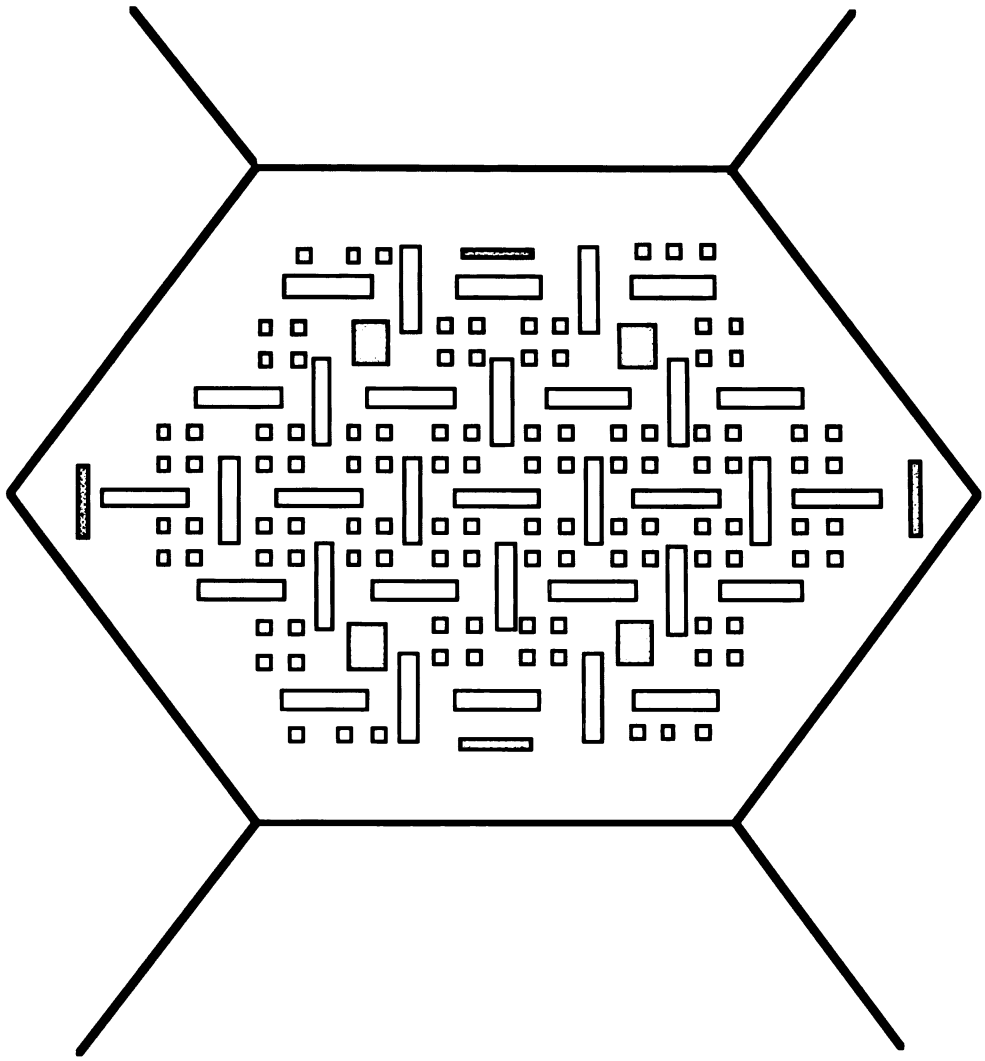


Figure 9.2.2 Photo Mask for the Design II Microfabricated Diffusion Membrane

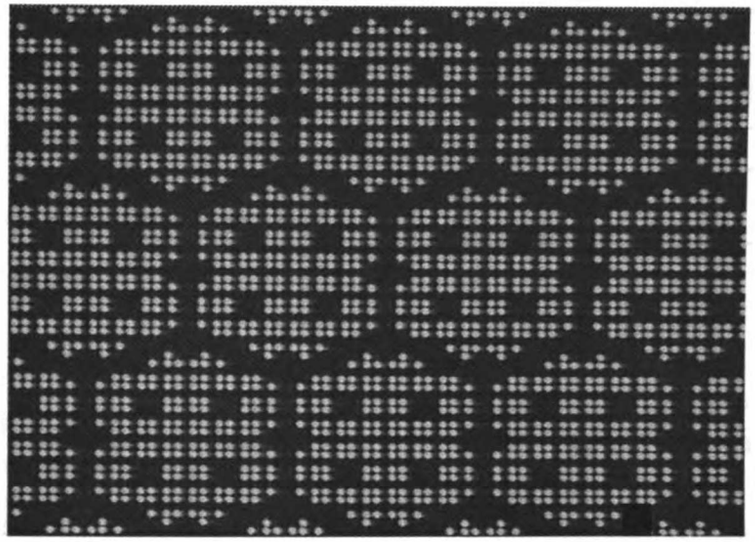


Figure 9.2.3 A micrograph of the Design II cell culture wafer. The etched holes on the filter membrane are 3 μm in diameter and the actual 78 nm diffusion channels are underneath.

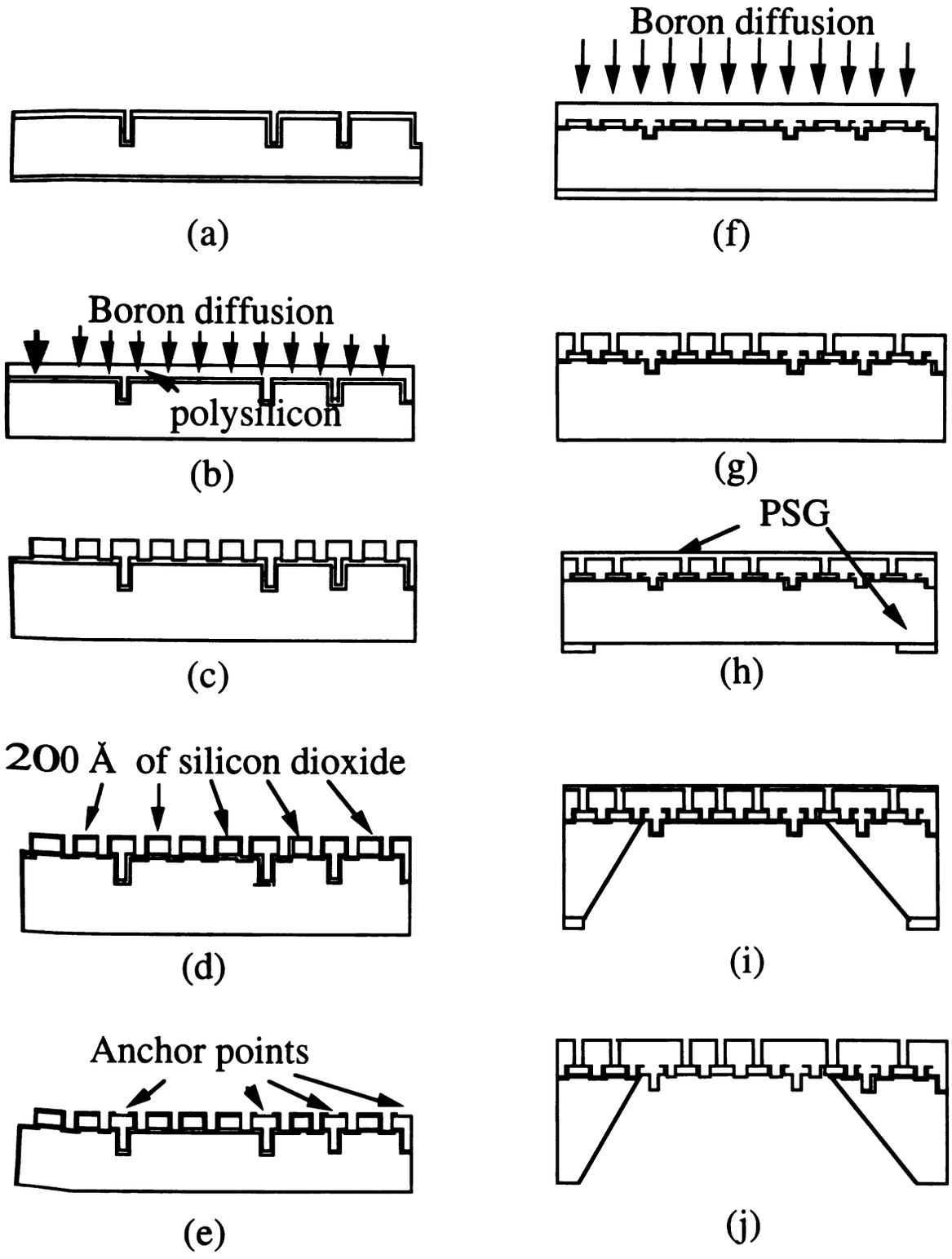


Figure 9.2.2 Fabrication Process Flow for Design II Biocapsule Membrane

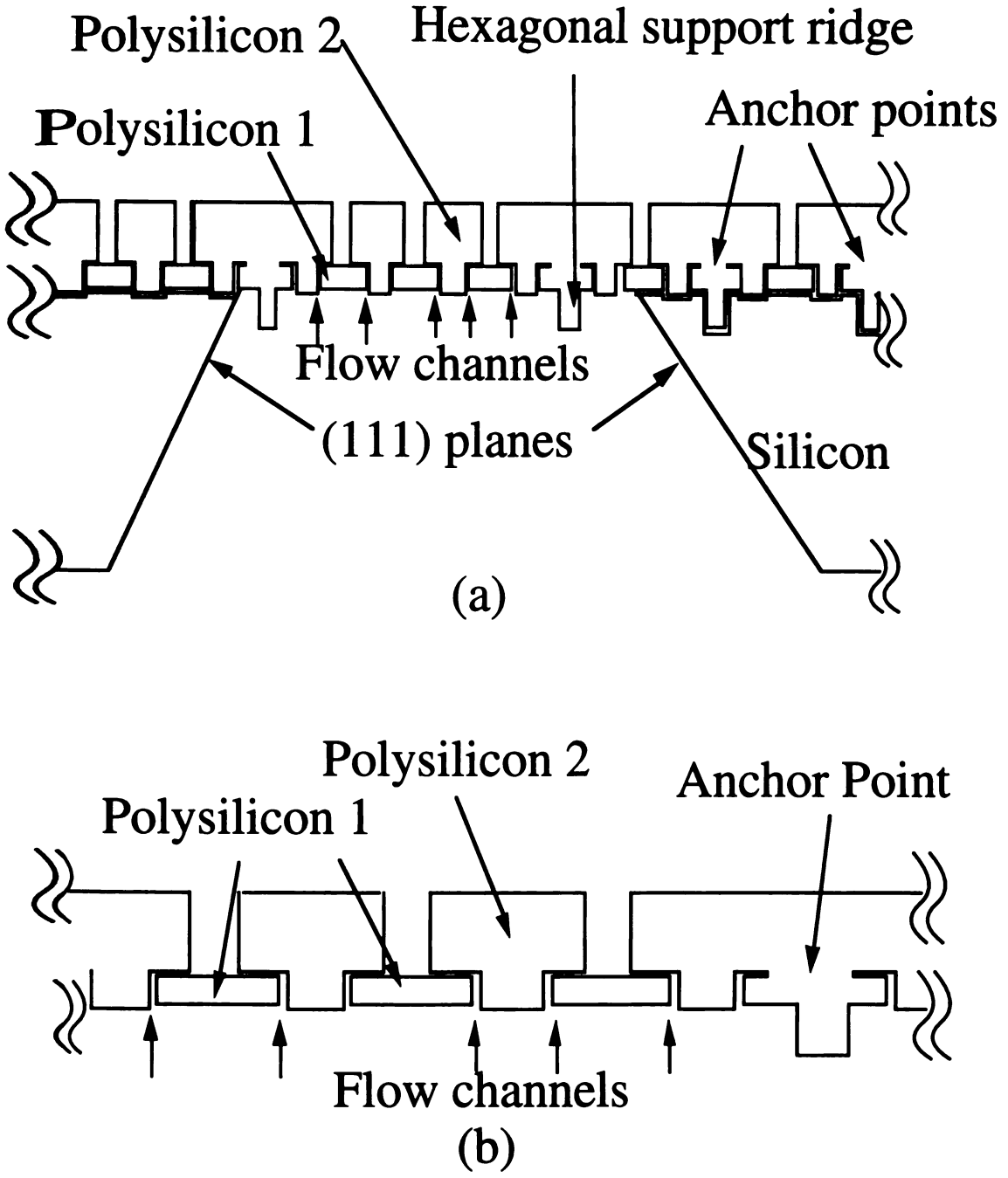


Figure 9.2.3 Schematic of Microfabricated (a) Half Biocapsule and (b) Diffusion **Membrane** of Design II

9.3 Full Biocapsule Fabrication.

After half biocapsules were sufficiently tested in vitro, the next step was to study full cell-containing biocapsules. In addition to Design II, another biocapsule design called M2 was developed and fabricated for the full microfabricated biocapsule biological studies. The fully assembled biocapsules consisted of two separate wafers bonded together with the desired cells contained within the microfabricated cavities on each of the wafers. The cavities containing the cells were bounded at the wafer surfaces by the microfabricated membranes. To achieve the fully assembled Design II biocapsule, two half biocapsules with Design II membranes, fabricated as described in the previous section, were bonded together. Similarly, two half biocapsules with Design M2 membranes were joined to form the fully assembled M2 Biocapsule. Although both biocapsule designs used the sacrificial oxide layer to achieve nanometer pore-sized microfabricated membranes, they differed in their pore length and geometric configuration (Figure 9.3.1).

The full fabrication protocol of Design M2 is described next (Figure 9.3.2). This biocapsule membrane is made of p^+ single crystal silicon, p^+ polysilicon and a sacrificial thin silicon dioxide layer. The first step in the fabrication of biocapsule membranes is the deposition of the supportive hexagonal ridges. Fabrication begins with growing a layer of silicon dioxide on a 4" (100) wafer, which is then photolithographically defined to produce supportive hexagonal trenches. A high temperature boron diffusion is done

on the silicon wafer. The etch area on the doped p^+ silicon is photolithographically defined using a photo mask containing a 2 μm x 2 μm array.

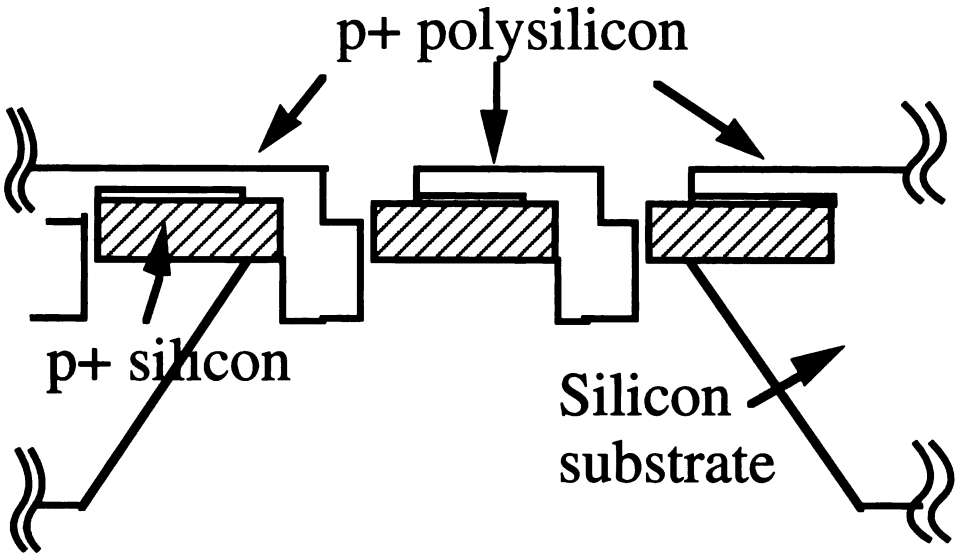


Figure 9.3.1 Cross sectional schematic of Design M2 biocapsule membrane **unit**.

A low temperature oxidation is used to generate a thin oxide layer of uniform thickness on top of the p^+ silicon. A one hour 900 °C low temperature dry oxidation is used to produce this sacrificial silicon dioxide layer. This oxide layer is removed in the final step and the thickness of layer determines the membrane pore diameter of the culture wafers and biocapsules. Again, the well-controlled oxidation environment can achieve a less than 5% thickness variation of the oxide layer over a 4 inch wafer.

As mentioned before, because the pore channels are made from a sandwiched silicon structure, anchor points are required to ensure the exact separation distance between the p^+ silicon and p^+ polysilicon layers. Several rectangular anchor points are defined on top of the thin oxide layer. The same photolithographic mask that is used to etch the p^+ silicon is used in defining the anchor points by offsetting the alignment 1 μm. A second polysilicon layer is then deposited and heavily doped using the previously described recipe. The second layer is anchored to the first deposited p^+ polysilicon layer through the defined anchor points.

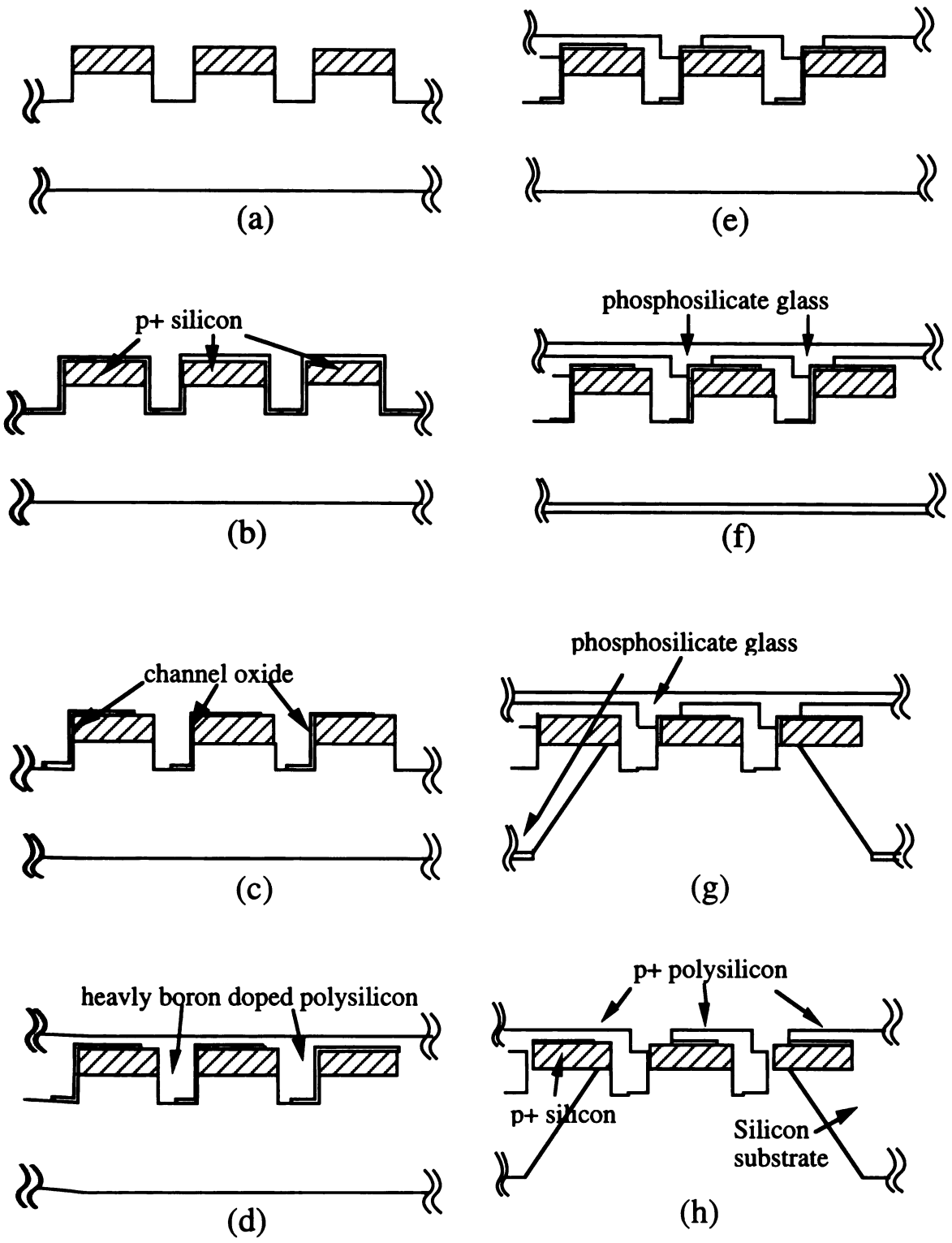


Figure 9.3.2 Fabrication Flow Process for M2 Membrane

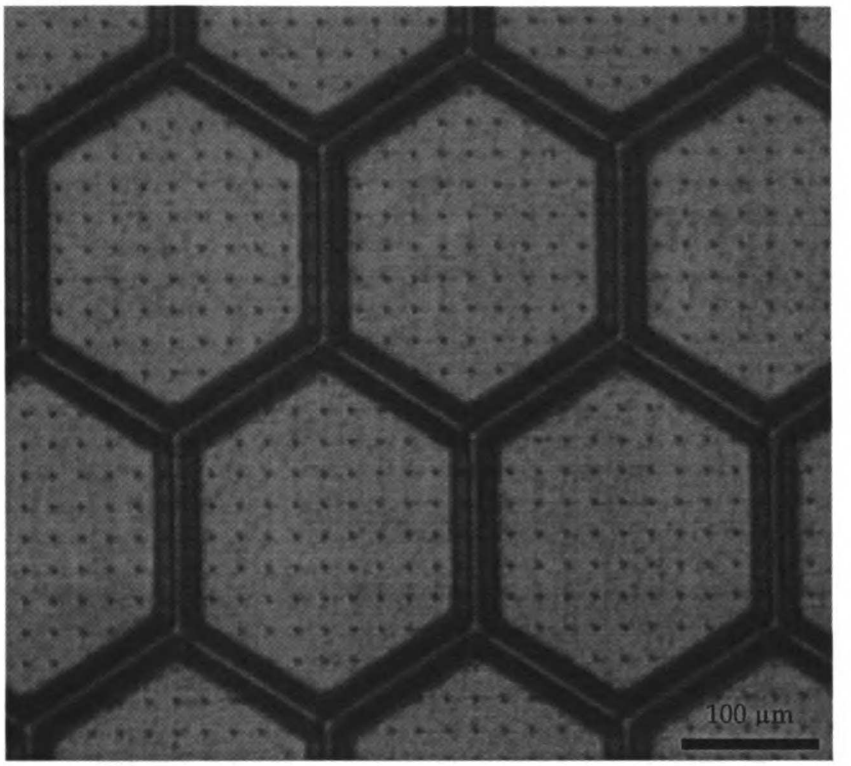


Figure 9.3.3 Micrograph of top surface of the M2 biocapsule membrane. Black dots are 2 x 2 µm entry ports.

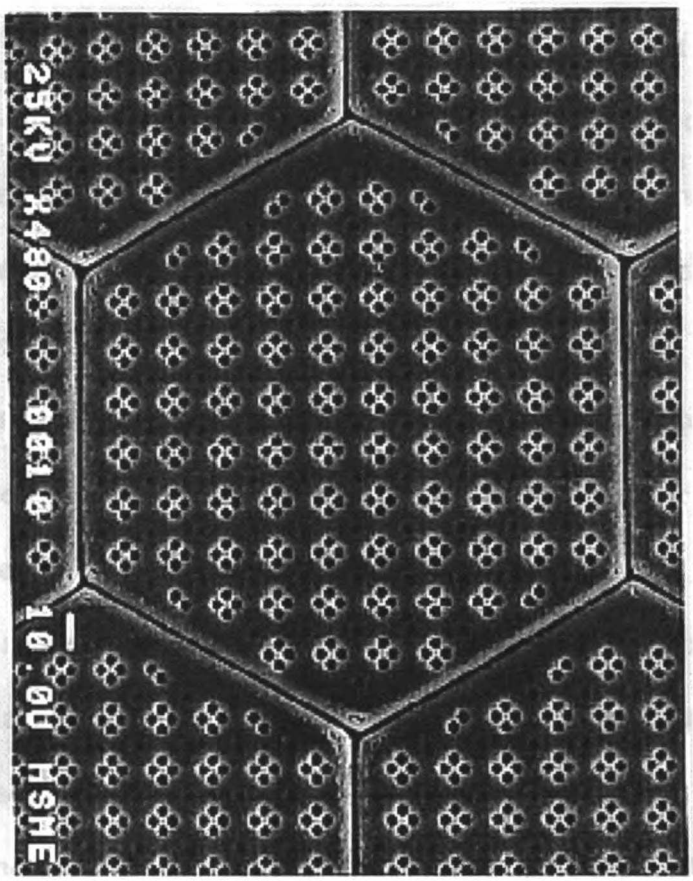


Figure 9.3.4 Micrograph of the Design M2 microfabricated diffusion membrane backside

A 3 μm layer of undoped polysilicon is deposited on top of the etched thin oxide layer, p^+ diffused, and wet oxidized to ease the removal of the BSG. The same mask is used again to produce the etch points on the p^+ polysilicon layer by offsetting the alignment vertically by 1 μm. Finally, etch windows are opened on the backside of the silicon wafer. The anisotropic silicon etching using EDP will stop automatically at the p^+ silicon and single crystal silicon interface. Buffered silicon dioxide etch is then used to dissolve silicon dioxide and PSG. This generates the pores for the biocapsules (Figures 9.3.3 and 9.3.4). Any particles or molecules suspended in the medium that have minimum rigid dimensions (d) larger than the thickness of the thin oxide layer (t) will not be allowed to diffuse through these channels (Figure 9.3.5).

Following membrane processing and anisotropic etching down to the membrane layer, a dicing step is performed to separate the die and create capsule halves. The desired cells can be placed inside each half-capsule, and the encapsulation is completed by bonding together two half-capsules (Figure 9.3.6).

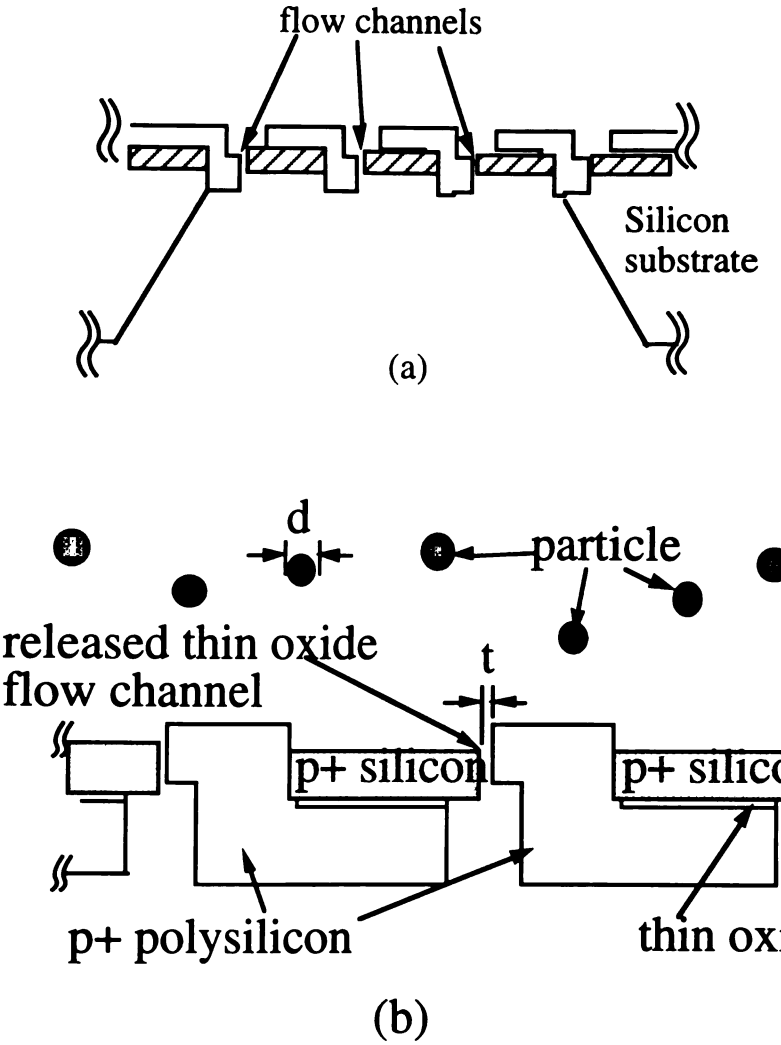


Figure 9.3.5 Schematic of permselectivity of microfabricated biocapsule membrane of Design M2

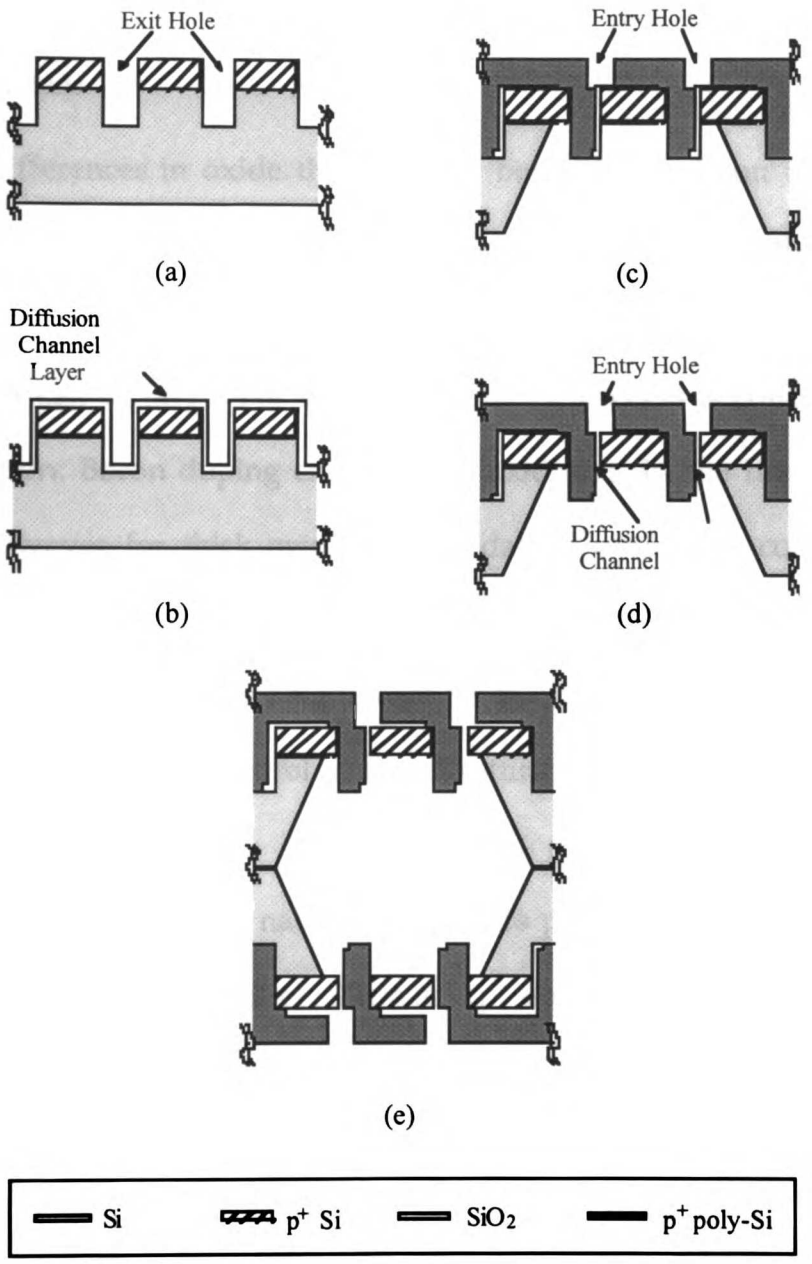


Figure 9.3.6 Essential fabrication steps for the microfabricated biocapsule (not to scale). (a) A 4" (100) Si wafer is first B-doped (>10^{20} cm^{-3}). 8 mm-deep exit holes are then etched through the p-doped region. (b) A 20-100 nm sacrificial oxide layer, which defines the diffusion channel, is deposited using dry thermal oxidation. (c) Anchor points are opened in the oxide and a 3 mm-thick, B-doped (>10^{20} cm^{-3}) polysilicon layer is deposited. A final etch step is performed to define entry holes in the polysilicon. (d) An EDP (ethylene diamine-pyrocatechol) etch is used to form the half-capsule. This will selectively etch the Si substrate without attacking the B-doped silicon and polysilicon layer, or the oxide. Following the etch, the channels are released with 5:1 buffered HF. (e) Two half capsules are bonded together to achieve the full biocapsule.

9.4 Channel Oxide

The differences in oxide thickness for both blank silicon and heavily boron doped silicon wafers have been examined in detail. For the case of a thin oxide (i.e. ~20nm range), the difference between pure silicon and p^+ doped silicon can be large. For thin oxidation, the process is controlled by a surface reaction. Boron doping enhances the oxidation in the linear reaction region. However for thick oxides, the oxidation process is controlled by diffusion, which is not changed by boron diffusion. Therefore, the oxide thickness difference is not very significant for thick oxides. Because of the fast initial oxidation rate, the control of the moisture in the oxidation furnace is very important.

At room temperature, the native oxide layers present after Piranha cleaning of the silicon wafer for both pure silicon and p^+ doped silicon are very similar. The thickness of the native oxide after piranha cleaning is around 11 to 13 Å, checked by ellipsometry. This native oxide does not change in a room temperature air environment and changes slightly (from 11 to 20Å after 50 days) if immersed in water. High temperature, high pressure, or steam sterilization via an autoclave does not change the native oxide, either.

Because the pore channels are made from a sandwiched silicon structure, anchor points are required to ensure the exact separation distance between the p^+ silicon and p^+ polysilicon layers. The same photolithographic mask that is used to etch the p^+ silicon is used in defining the anchor points.

The anchor points are defined by offsetting the alignment upward by 1 μm as shown in the finer dashed square in Figure 5(a). The defined area is then plasma etched to remove the thin channel oxide. Due to the offset and later silicon dioxide etch, there will be no oxide in the exposed anchor point areas. A layer of 3 μm undoped polysilicon is deposited on top of the etched thin oxide layer, p^+ diffused, and wet oxidized to ease the removal of the BSG as previously described. The same mask is used again to produce the etch points on the p^+ polysilicon layer by offsetting the alignment downward by 1 mm (Figure 5(a)). Because of the thin oxide in between the p^+ polysilicon layer and p^+ silicon layer, the overetch into the p^+ silicon layer can be alleviated if a highly selective silicon plasma etch recipe is used. A slight overetch into the underlying p^+ silicon layer will not effect the performance of the membrane, since it does not change the oxide channel dimensions. The wafers are then wet oxidized at 1000°C for 1 hour and LPCVD is used to deposit a 2 μm PSG layer to protect the membrane against later EDP etching. Backside etching squares (2 μm x 2 μm) are defined and the device wafer is EDP etched at 100°C for about 10 hours. Buffered silicon dioxide etch is used to dissolve silicon dioxide. Finally, the device wafer is cleaned and rinsed as described previously.

The completion of the silicon dioxide removal can be confirmed by checking the interference pattern on the plasma etched p^+ silicon membrane side. If the diffusion channels are etched completely through, a dark reflection square is observable at the cell culture well position. This is due to

the capillary effect of the water inside the diffusion channels. These dark patterns cannot be found when there are no diffusion channels. The capillary effect is very prominent in this dimensional range.

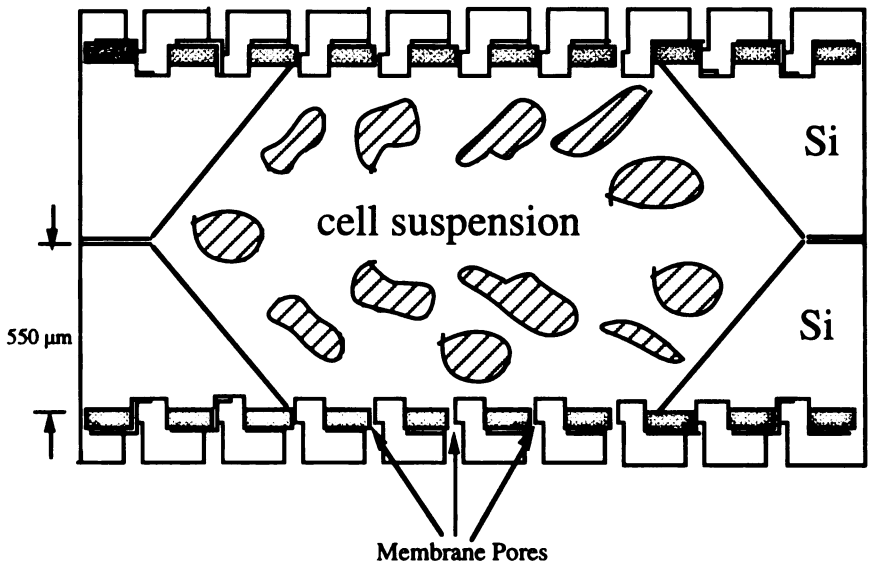


Figure 9.4.1 Schematic of fully assembled biocapsule containing cells

9.5 Assembly Issues

One major difficulties associated with the microfabricated biocapsule is the procedure for bonding the two half capsules together. Due to the close proximity of cells and living tissue to both the internal and external capsule interface, the choice for a suitable bonding agent is nontrivial.

Because the cells are placed in the biocapsule before the pieces are bonded together, the bonding process must be able to occur in the presence of the biological materials to be transplanted without affecting their functionality. While adhesives have been developed that can be used in biological conditions, especially for dentistry and surgery, there has been little work done on bonding techniques that are specifically non-cytotoxic during the application and curing process. Additionally, the introduction of silicon as the biocapsule material increases the limitations of the bonding technique employed. The surface chemistry of silicon is quite different from traditional biomaterials and therefore, a biocompatible bonding agent for silicon is not necessarily readily available, nor thoroughly investigated.

The microelectronics industry has studied adhesives for standard silicon processing, as needed for the packaging of ICs and Multi-Chip Modules (MCMs). However, the conditions for bonding in these applications is not similar to those needed for biohybrid devices. In addition to typical mechanical requirements, including the strength of the bond, the resistance to shear stresses (capsules in the body are subjected to minimal tensile stresses),

there are several additional constraints in biomedical applications. Important parameters such as bonding temperature, residual solvents, outgassing, degradability, impermeability, and chemical composition must be taken into consideration when choosing an appropriate bonding agent for our biocapsule.

In our in vitro studies with fully bonded microfabricated biocapsules, the bonding agent used was a medical grade silicone elastomer (Type A Medical Adhesive, Factor II Inc.). However, because this is not an ideal bonding agent, studies have been and are currently being performed on the suitability of other bonding alternatives including industrial grade silicone, medical grade silicone, Polymethylmethacrylate (PMMA), Polypropylmethacrylate (PPMA), and PEMBA [Hansford, 1996]. The materials investigated were selected because of their processing temperatures, their previous use (or previous use of materials similar to them) as adhesives, and other qualitative factors (e.g. response to initial testing). Most other traditional materials that have been used to bond silicon wafers are not acceptable due to their processing temperatures, outgassing problems, or presence of other potentially cytotoxic substances. Other biomedical adhesives were not suitable due to difficulty in handling or cytotoxicity.

The results obtained from both mechanical and cytotoxicity testing show that lower T_g methacrylates (PPMA and PEMBA) could make suitable bonding agents for the biocapsules [Hansford, 1996; Hansford, Desai, and Ferrari, *Unpublished results*]. Both of these adhesives exhibited non-toxicity cell culture studies. Preliminary studies have shown that these adhesives can be used to

bond silicon at body temperature and that bonding at higher temperatures is also possible. It was found that bringing the polymer-coated silicon in contact with the filled wafer at a higher temperature (70 °C) only raises the temperature of liquid in another silicon cavity by 4-5 °C. It has been shown that highly sensitive cells can survive temperatures as high as 45 °C for periods up to two minutes [Craig, 1993].

The methacrylates have been shown to have sufficient mechanical properties for use in the body once properly bonded. Both PPMA and PBEMA have higher adhesive strengths that PMMA, which is currently used as a bone cement, one of the most demanding mechanical applications of biomaterials. In addition, since our application is for small passive *in vivo* devices, the overall adhesive requirements are well below the strengths of most bonding processes.

10. Microfabricated Biocapsule Material Biocompatibility

10.1 In Vitro Biocompatibility Testing of Microfabricated Silicon Surfaces

Aside from the many fabrication challenges, several other issues needed to be considered in the development of the microfabricated biocapsule. Most importantly, interactions between the capsule and the cells contained in the capsule had to be characterized. It was necessary to create an environment in which the encapsulated cells could remain stable and viable in order to perform their intended metabolic function. Therefore, capsules were fabricated with pore sizes that were large enough for the timely diffusion of necessary cell nutrients and oxygen. Furthermore, it was critical to make sure that the capsule did elicit excessive inflammatory or chronic reactions from the host when in contact with the biological environment. Fibrotic encapsulation could not reduce pore patency below a limit of proper functionality. Throughout the course of this research, several of these issues were addressed and overcome.

10.1.1 Biocompatibility Testing Methods

Extensive literature searches revealed there were few studies on the behavior of silicon structures in the body or the overall bio-toxicity of silicon. Recently there have been some studies on growing certain cell types on silicon substrates with varying microtopographical features. However, these studies do not adequately address the effects of silicon on cellular viability,

proliferation, and function [Brunette, 1995]. Therefore, the biocompatibility of the proposed silicon microimplants had to be verified before continuing studies on feasibility and effectiveness of silicon biocapsules as biomedical devices. To this end, tests were conducted, as part of this thesis, to determine the *in vitro* and *in vivo* biocompatibility and cytotoxicity of silicon microimplants. These studies were conducted using established protocols for biomaterials testing.

For the purposes of this research, biomaterial toxicity was assessed by examining cell- material interactions in terms of cell morpholgy, growth, and function when in contact with the test material. All initial tests were carried out using a "prototypical" test culture wafer (Figure 10.1.1). This culture wafer array consisted a quarter silicon wafer containing a series of 1 mm by 1 mm wells of 0.5 mm depth. Each well was bounded by a bottom membrane with hexagonally arranged pores of 3 micron diameter. Each culture wafer was supported above tissue culture medium with autoclavable tygon tubing. Thus, the culture medium could only reach the cells in the wells by diffusion through the bottom membranes of the culture wafer. These culture wafers served as an *in vitro* test model of the proposed biocapsule, in order to more fully characterize cell - biocapsule interactions.

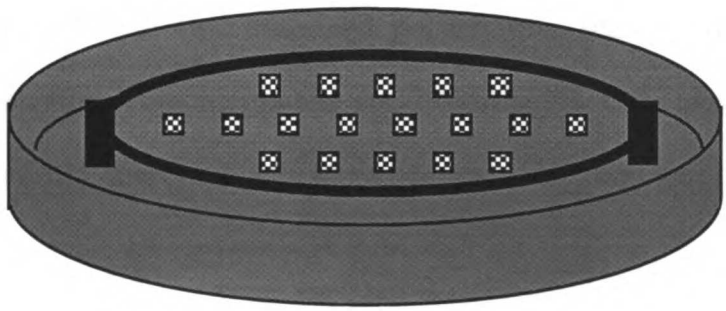


Figure 10.1.1. Prototypical culture wafer for in vitro biocompatibility testing

Using cultured HeLa cells (derived from human cervical carcinoma cells), suspension of cells were incubated and cultured inside the 1 mm wells of the silicon culture wafer. Microfabricated culture wafers were raised 0.5 cm with the tube supports in a standard tissue culture dish so that nutrients could flow underneath the membrane bound wells. To each dish, 20 ml of DMEM + 10% FBS culture medium was added. To each well, 5 µl of HeLa cell suspension was added (6.3 $x10^5$ cell/ml). The culture wafers were incubated at 37°C in a 5% CO_2 atmosphere. Cellular viability and proliferation within these microfabricated wells were monitored over a period of 24 to 96 hours. Cells were subsequently pipetted out of the wells and counted via a hemocytometer. Staining with both exclusion and inclusion dyes (trypan blue and methylene blue, respectively) was used to assess the viability of cells and relative cytotoxicity of the material.

Rat alveolar macrophages (ATCC) were also cultured within microfabricated culture wafers. A 10 ml cell suspension in RPMI medium with 10% FBS was seeded at a density of $4.5x10^5$ cell/ml on four microfabricated

substrates, as well as on two standard polystyrene tissue culture dishes (90 mm). The substrates were incubated at 37°C in a 5% CO_2 atmosphere for 72 hours. Subsequently, attached cells were examined, typsinized, and counted.

10.1.2 Data and Results

HeLa cells were observed both inside the microfabricated wells and on the adjacent surfaces of the microfabricated culture wafers using a light microscope. An increase in cell population was observed daily within the wells. HeLa cells had normal morphology when in contact with all microfabricated surfaces. Cell viability was found to be greater than 90% over the course of four days, similar to that of HeLa cells in standard polystyrene culture dish controls (Figure 10.1.2).

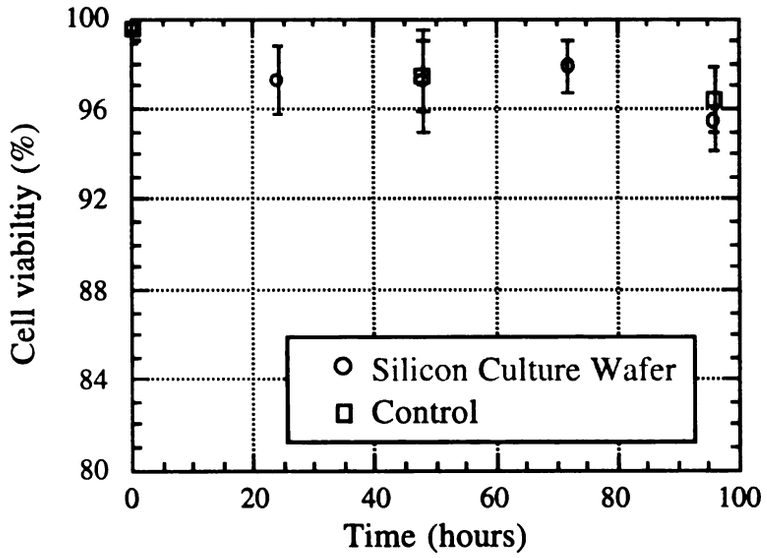


Figure 10.1.2 HeLa cell viability within microfabricated silicon culture wafers in relation to viability in standard polystyrene culture dishes

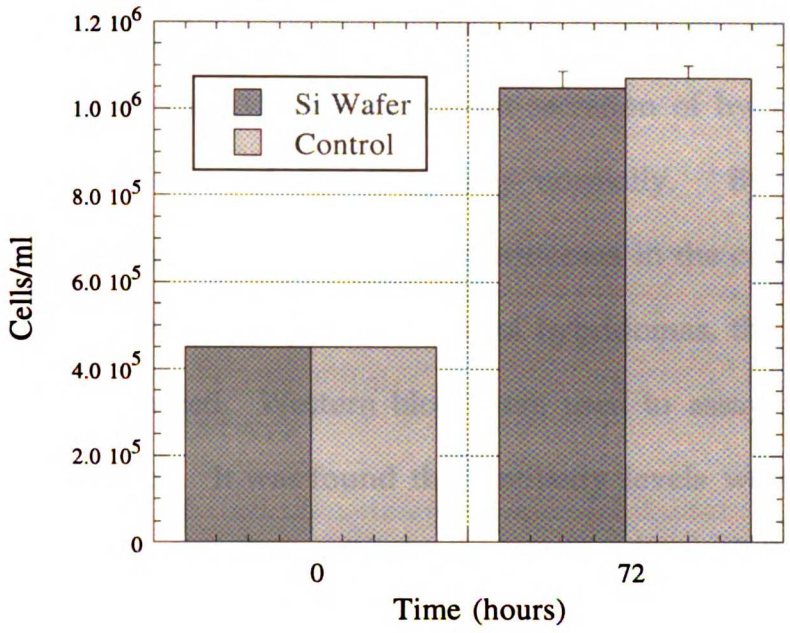


Figure 10.1.3 Macrophage growth within silicon culture wafers and control polystyrene culture dishes.

Over a period of 72 hours, macrophages were monitored to detect any morphological changes or damage. Macrophages adhered normally on all microfabricated silicon surfaces. They displayed confluency on both the silicon surfaces and the control polystyrene surfaces. On both substrates, cells were round and shiny in appearance, indicative of normal morphology. Counting of the macrophages revealed that the macrophages on the microfabricated culture wafer doubled in population to $10.5\pm.385\times10^5$ cell/ml (mean±SD) in 72 hours. Similarly, control macrophage populations grew to $10.7\pm..295\times10^5$ cells/ml (Figure 10.1.3)

10.2 Hybridoma functionality

Additionally, silicon culture wafers were tested for any toxic effects on cellular function by monitoring the protein secretion of hybridomas. These cells secrete antibodies when functioning normally. By measuring the concentration of antibodies secreted by hybridomas in the culture wafer wells and outside of the wells compared to control hybridomas, the functionality of cells could be compared. Western blots were used to assay the presence of antibodies in solution. It was found that antibody levels were quantitatively similar in the culture wafer wells, in the surrounding medium, and in the control sample. This indicated that no impairment of hybridoma function occurred due to the cellular contact with the silicon membrane.

10.3 In Vivo Biocompatibility Tests

As noted, several of the previously published microdevice-related studies have focused on the effects of silicon-based electrode arrays in cortical tissue [Edell, Churchill, and Gourley, 1992; Stensaas and Stensaas, 1978]. Electrodes using silicon micromachining technology were shown to be biocompatible in both peripheral and central nerves of the cerebral cortex. However, biocompatibility, in these cases, was defined in terms of neuron survival. These studies focused on issues of structural shape and method of implantation rather than the material-tissue interaction itself. With the surgical assistance of Dr. Giuseppe Mazzoni, Professor of Surgery at Mercy Hospital Microsurgical Institute in San Diego, the long-term biocompatibility, in terms

of tissue response and changes in material properties, of silicon-based implants was evaluated in several different tissue sites. The biostability and bio-degradation properties of the implant material were examined. The simple test silicon microimplants employed were designed to simulate features of relevance for future microfabricated biocapsule technology [Desai, Mazzoni, and Ferrari, 1995].

10.3.1 Materials and Methods

Two types of microimplants were fabricated and tested for biocompatibility. Type A microimplants consisted of single crystalline silicon blocks, coated with polysilicon on both sides. The blocks of material were fabricated into 3 mm by 3 mm squares having a thickness of .5 mm, with blind filtration channels (2 μm in diameter) cut into the central portions of each square, by methods of photolithography. Type B microimplants were single crystal silicon blocks, also 3 mm by 3 mm by .5 mm, with no polysilicon coating. Whenever exposed to air or aqueous environments, silicon develops a very thin surface oxide layer. Such a layer, a few nanometers in thickness is expected to have formed on the surface of both implant types. The described implants were reproducible in size and shape, fabricated with the outstanding degree of precision afforded by current microfabrication procedures.

Implantation and Recovery

Two non imbred rats, weighing 200 grams each, were used to evaluate the biocompatibility of the silicon microimplants. The rats were anaesthetized with ether and routine laparotomies were performed. Rat #1 received Type A

silicon implants inserted into both the liver and pancreas. Type B silicon implants were inserted into small pouches made in the spleen, liver, pancreas, and kidney of Rat #2.

These silicon structures were implanted for a period of seven months. This length of implantation was sufficient to encompass both acute and chronic tissue inflammatory response. At seven months, the animals were anaesthetized with ether. A second laparotomy was performed. The spleen, liver, pancreas, and kidney of Rat #2 was removed and fixed in formalin (12%). The liver and pancreas of rat #1 was also removed and fixed with formalin (12%). The explanted tissue from both animals was sectioned and stained for routine histology with hematoxylin and eosin. The implants were also removed and stored in alcohol until further analysis.

10.3.2 Data and Results

Explanted silicon squares were examined under both reflected light microscope and scanning electron microscope. For both groups of silicon microimplants, the surface appearance was the same both before and after the implantation (Figure 10.3.1). There appeared to be no changes in the mechanical or surface properties of the implants. No corrosion was observed. The surface of the samples remained smooth although some of the wafers displayed slight tissue adherence (Figure 10.3.2). The filtration pores appeared clear and free from any obstructions or biological debris. The edges of the squares were rough as a consequence of the initial sanding and preparation before implantation.

Tissue Response

No gross abnormalities of color or consistency were observed in the tissue surrounding the implant (Figures 10.3.3 - 10.3.6). The tissue response to the implants was assessed under light microscopy using modification of the method developed by Salthouse (1983). Tissue samples were assigned a cellular response grade (Table 10.3.1) depending on the type and concentration of cells in the reaction zone area and the thickness of the fibrous capsule, if formed. Grades 1-5 were assigned for capsule thickness: Grade 1(0-100 μm zone); Grade 2 (100-300 μm zone); Grade 3 (300-500 μm zone); Grade 4 (500-1000 μm zone); grade 5 (1000-2000 μm zone).

Cellular concentration in reaction zone was graded from 0-5 (table 10.3.1). Cell type was graded as follows: Neutrophils = 5, Giant Cells = 2, Macrophages, Fibroblasts, and Lymphocytes = 1. The final reaction grade (FRG) was calculated by the following equation: (Thickness x 5) + (Concentration x 3) + (Cell Type x 5) = final score and the final numerical score was expressed descriptively as : no reaction (0); minimal (1-10); slight (11-25); moderate (26-40); marked (41-60); extreme (60+).

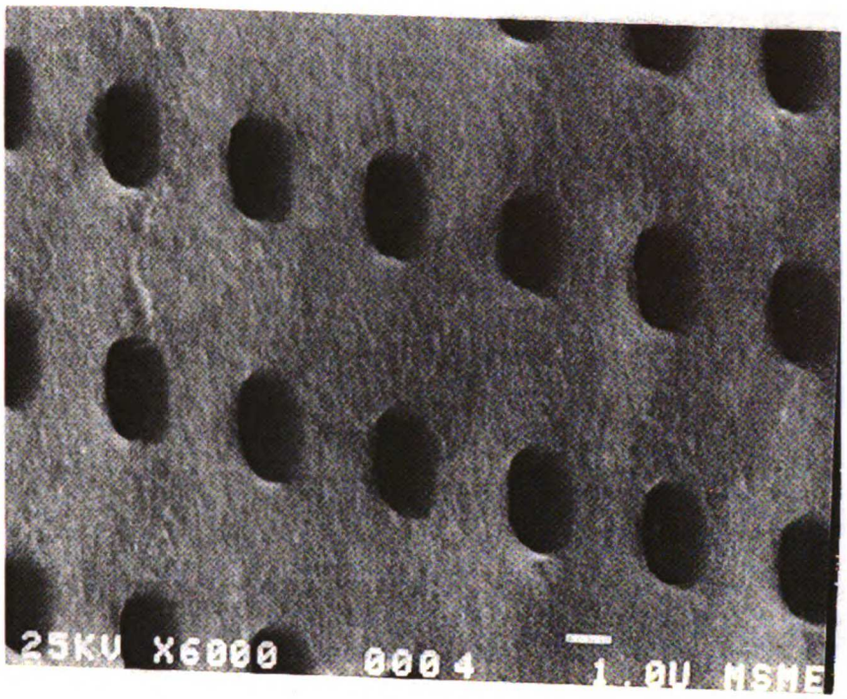


Figure 10.3.1 Blind filtration pores of implanted silicon microimplants (Type A)

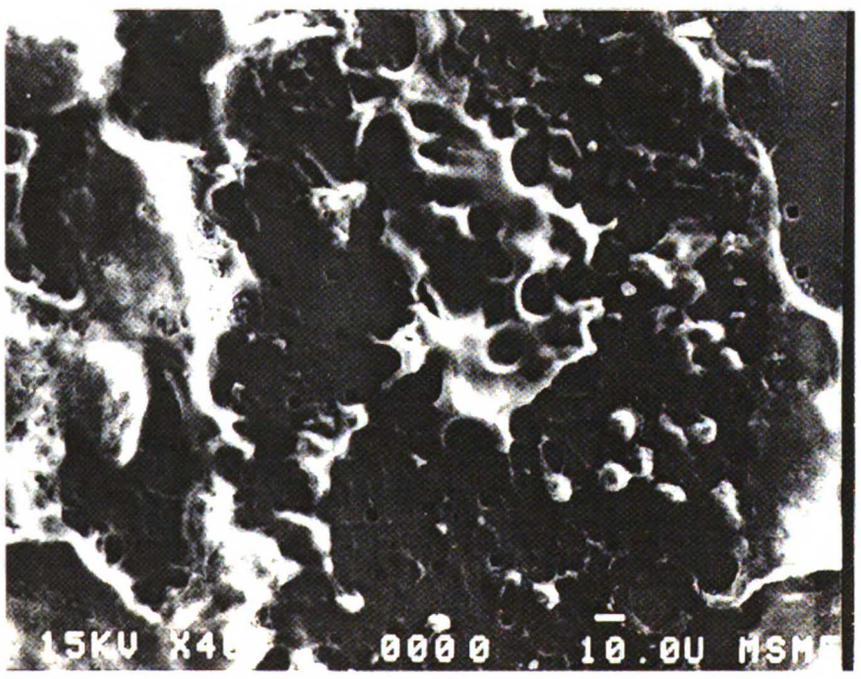


Figure 10.3.2 Loose tissue adhesion to silicon microimplants

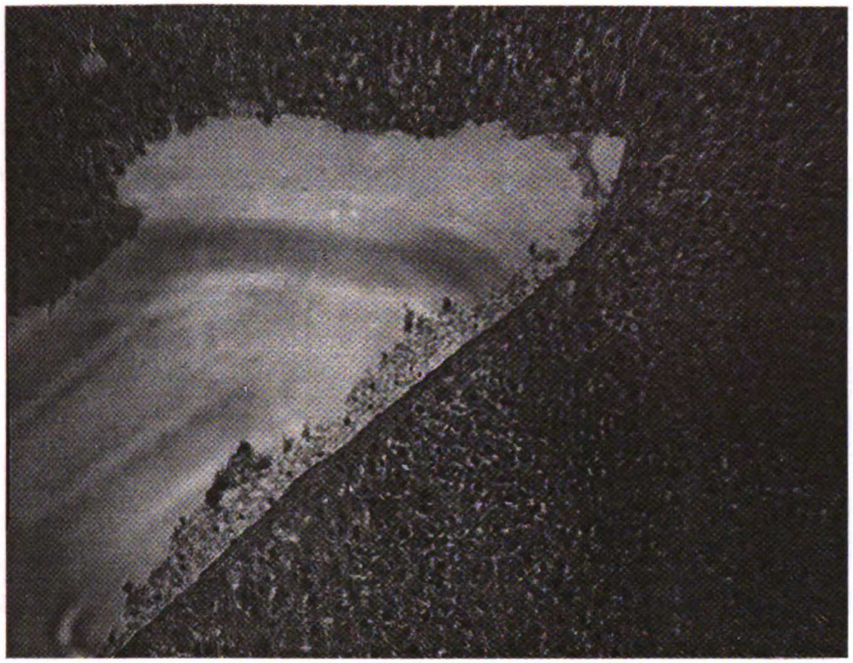


Figure 10.3.3 Spleen tissue surrounding silicon microimplant (Type A; 10x)

Figure 10.3.4 Spleen tissue surrounding silicon microimplant (Type A; 100x)

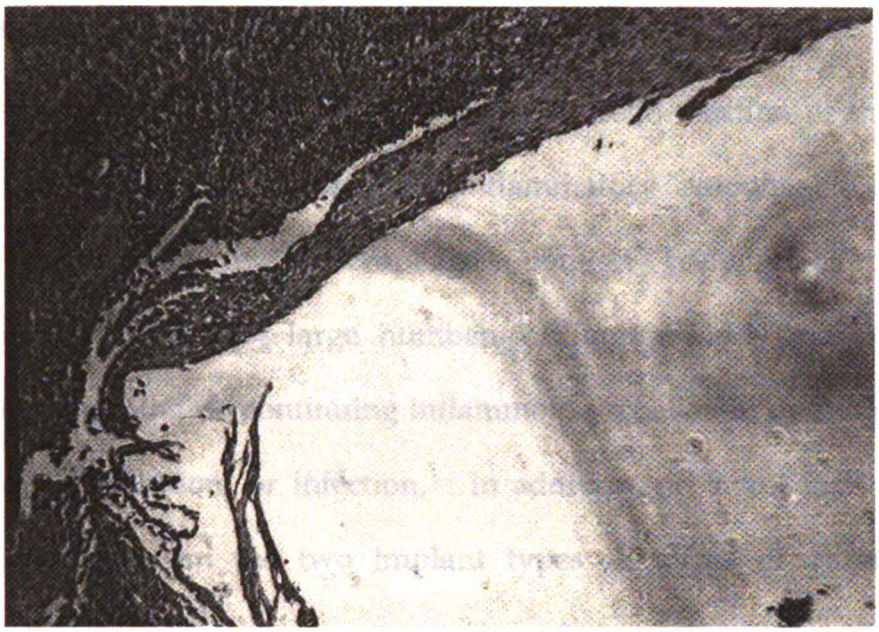


Figure 10.3.5 Spleen tissue surrounding silicon microimplant (Type B; 100x)

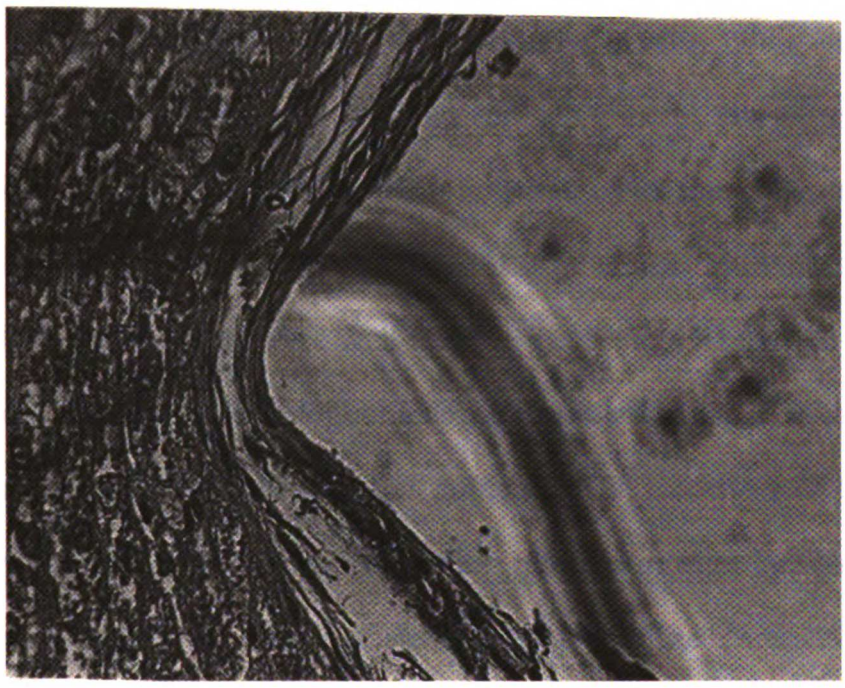


Figure 10.3.6 Liver tissue surrounding silicon microimplant (Type B; 63x)

10.3.3 Discussion

Histopathological data indicated that the tissue reaction at the implant site progressed from an initial acute inflammatory response to a chronic response marked by few macrophages, foreign body giant cells, and fibroblasts. The absence of large numbers of foreign body giant cells and neutrophils suggested no continuing inflammatory challenge due to corrosion, degradation, dissolution, or infection. In addition, no significant difference was observed between the two implant types in terms of chronic tissue response.

A fibrous tissue capsule was maintained by the continuing presence of the implant. Generally, the fibrous capsules surrounding the implants had a thickness of less than 300μm after seven months in situ, while surrounding tissue appeared normal and well vascularized. In general, the fibrous capsule was well-formed and displayed little migration. No necrosis, calcification, tumorgenesis, or infection was observed at any of the implant sites. This suggested that the implants were well-tolerated and most likely non-toxic.

Fibrous capsules had a dogbone configuration mostly due to the sharp corners presented to the tissue by the implant. The thickness of the fibrous capsule is related to several factors including relative motion between implant and tissue, shape of the implant, and surface features of the materials. Thus, the moderate reactions that did occur were probably present due to the rough edges and corners of the silicon microimplants. By smoothing out these edges, the reaction could be further minimized. The specific geometries and

dimensions of the implanted test structures were chosen so as to furnish relevant information for the feasibility of implanting microfabricated biocapsules.

Table 10.3.1 Tissue response grades [modified from Salthouse et al., 1983]

	Thickness	**Concentration**	**Cell Type**	**Final Grade**
Rat 1 (Type A)				
Pancreas 1	1	0	0	5
Pancreas 2	1	0	0	5
Liver 1	3	1	1	23
Liver 2	1	1	1	13
Rat 2 (Type B)				
Spleen 1	1	0	0	5
Spleen 2	1	1	1	13
Kidney 1	2	1	1	18
Kidney 2	3	2	1	26
Liver 1	2	2	1	21
Liver 2	1	1	1	13
Pancreas 1	1	0	0	5
Pancreas 2	1	1	1	13

10.3.4 Conclusions

Polymers employed in current microencapsulation research have been found to be moderately toxic when implanted in vivo for up to one month [Colton, 1995]. By comparison, our results seemed to encourage the use of silicon-based microcapsules, with a number of caveats. First, the host tissue response would presumably vary as a function of the type and amount of encapsulated donor tissue. Furthermore, in a cell encapsulation device, the pores in the diffusion-barrier portion of the capsule surface would have to be

much smaller in dimension than the pores of the implanted test microstructures. This could change the observed tissue response. However, it was estimated that pores in the cited diffusion barriers should be in the nanometer range, which would render the microcapsule surface essentially solid and smooth to the surrounding tissue and cells. Given the biocompatibility of the solid implants (type B) it may then be concluded that the microcapsule walls will not elicit a stronger host tissue response than these test microimplants.

It was envisioned that the overall microcapsule dimensions would be in the 100μm to millimeter range. In this sense, the dimension-related biocompatibility results associated with the millimeter-sized implants discussed could apply to the microimplant walls, which for the diffusion-barrier part of the microcapsule need be very thin (100 nanometer range) to allow sufficiently rapid insulin diffusion rates.

In broad terms, the silicon-based microimplants were shown to be non-toxic towards tissue, non-degradable, and with a sufficient degree of biological inertness to warrant further investigations into the use of silicon based microfabricated biocapsules for cell transplantation and implantation.

10.4 Microfabricated Surface Modification Studies

While studies on silicon have shown it to be generally biocompatible, we sought to investigate ways to further improve its biocompatibility in view of its potential applications in diffusion-based hybrid organs [Zhang, Desai, and Ferrari, 1998]. The negative charge of untreated silicon/silica surfaces can sometimes lead to increased surface-protein interactions and adsorption. This protein adsorption may ultimately reduce biocapsule biocompatibility and functionality by negatively influencing cell-surface interactions or clogging membrane pores.

Although surface modifications techniques to reduce protein adsorption and cell adhesion has been extensively investigated for polymeric biomaterials, significantly less is known about the modification of silicon surfaces. Surface modification to achieve enhanced biocompatibility is commonly achieved through chemical coupling or physical adsorption of bioinert polymer chains. Hydrophilic polymer coatings consisting of poly ethylene oxide (PEO) or poly ethylene glycol (PEG) have been used successfully to modify several different biomaterials.

Polyethylene glycol, a water soluble, non-toxic, and non-immunogenic polymer, has been shown to significantly decrease both protein and cell adsorption on several biomaterials [Llanos and Sefton, 1993; Aldenhoff, 1995]. The coating of PEG films can be done by physisorption, graft polymerization, or covalent coupling techniques. Using chemical coupling techniques developed by Zhang and Ferrari (1997), the interaction of surface modified

silicon with biological cells was observed. The adhesion of cells to a given biomaterial and subsequent proliferation is dictated by initial protein adsorption. A decrease in protein adsorption most often leads to a decrease in cellular attachment. The activation of collagen specific receptors upon cell adhesion to an implant surface is thought to lead to collagen deposition and subsequent walling off the implant by an encapsulating fibrous layer.

10.4.1 Materials and Methods

The attachment and growth of human fibroblasts and HeLa cells were studied by a cell culture method to evaluate the biocompatibility and cell adsorptive properties of the modified silicon surfaces. Fibroblasts and HeLa cells were chosen in view of their role in the fibrotic response and reproducible growth profiles in culture, respectively.

PEG was immobilized on silicon by the functionalization of a PEG precursor containing SiCl3 groups at its chain ends, followed by reaction of surfaces with the compound PEG-OSiCl3. The silanols formed after hydrolysis of the compound condense with silanols on silicon surfaces, leaving the silicon surface modified with PEG moieties [Zhang, Desai, and Ferrari, 1997].

Cell adhesion properties of PEG coated and uncoated silicon surfaces were evaluated in vitro by cultivating two cell lines, a finite cell line of human lung fibroblasts (IMR 90) and a continuous cell line of human epithelial cells (Adherent HeLa) on these surfaces. Both cell lines are anchorage dependent.

Cell Culture Conditions

Human fibroblasts were grown in Dulbecco's Modified Eagle Medium (DMEM, Gibco) supplement with 10% Fetal Bovine Serum (Hyclone, Logan, UT) in a humidified incubator with 5% CO2. The fibroblasts were removed from tissue culture flasks by incubating in 0.05% trypsin/0.6 mM EDTA for five minutes. HeLa cells in DMEM + 10% FBS were harvested from a T-flask. Cell suspensions were counted in a particle counter (Coulter) equipped with a 100 μm diameter orifice. Cells were then reseeded on modified and unmodified silicon surfaces for either cell attachment or cell growth monitoring.

Cell attachment assay

The number of cells attached to modified and unmodified silicon substrates was determined at the time intervals of 30, 120, 240, and 300 minutes. For both cell lines, approximately 3x105 cells in 0.5 ml culture medium containing 10% FBS were pipetted into each well of 24-well tissue culture plates containing the modified and unmodified silicon substrates. After incubation, the sample substrates were transferred to empty culture plate wells and rinsed once with 1 ml PBS to remove unattached cells. Substrates were then incubated with 0.2 ml 0.05% trypsin/EDTA to remove attached cells. Trypsin was subsequently neutralized with 0.3 ml DMEM + 10% FBS. Cells detached from the substrates were counted twice with a particle counter and also a hemocytometer.

Cell proliferation assay

The proliferation of attached cells was determined at 1, 2, and 4 days. Cells on the substrates were detached by trypsinization and counted as described above. Cell suspensions of 3x105 in 0.5 ml of medium with 10% FBS were cultured in 24 well tissue culture plates containing the substrates in a humidified incubator for up to 4 days. Two sets of identical cultures were plates for each experiment, since each complete experiment included both an attachment and growth assay.

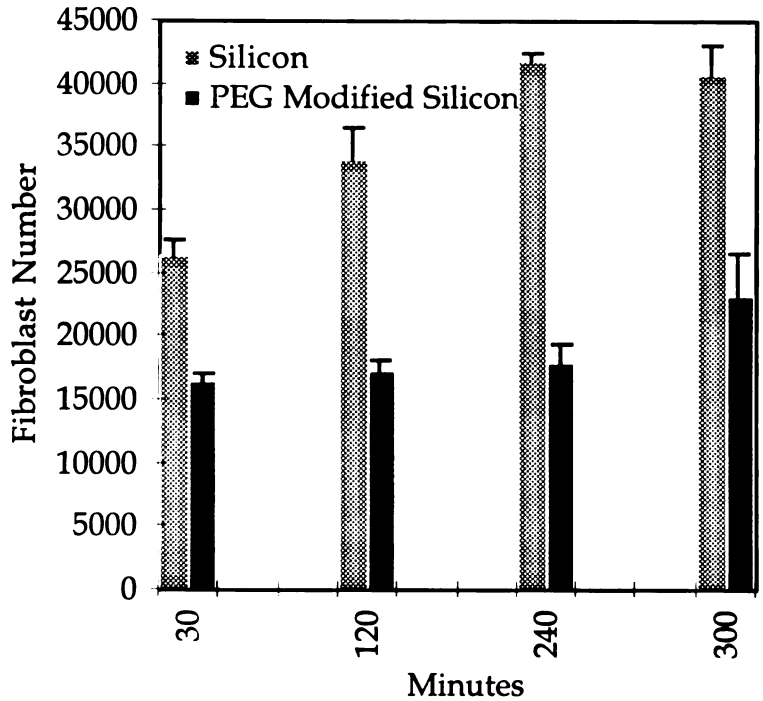


Figure 10.4.1. Adhesion of human epithelial fibroblast cells to untreated hydrophilic silicon and PEG modified silicon.

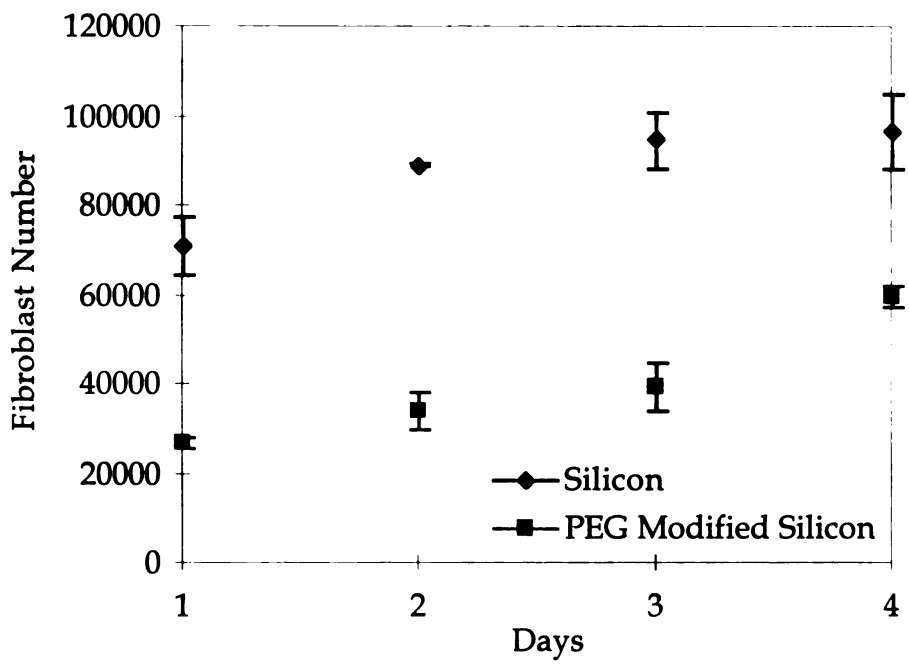


Figure 10.4.2 Proliferation of human epithelial fibroblast cells to untreated hydrophilic silicon and PEG modified silicon.

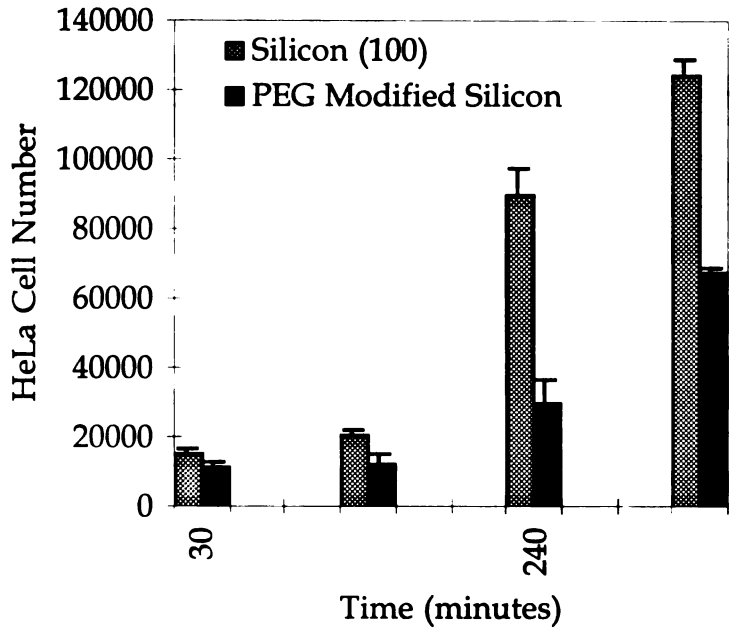


Figure 10.4.3 Adhesion of HeLa cells to untreated hydrophilic silicon and PEG modified silicon.

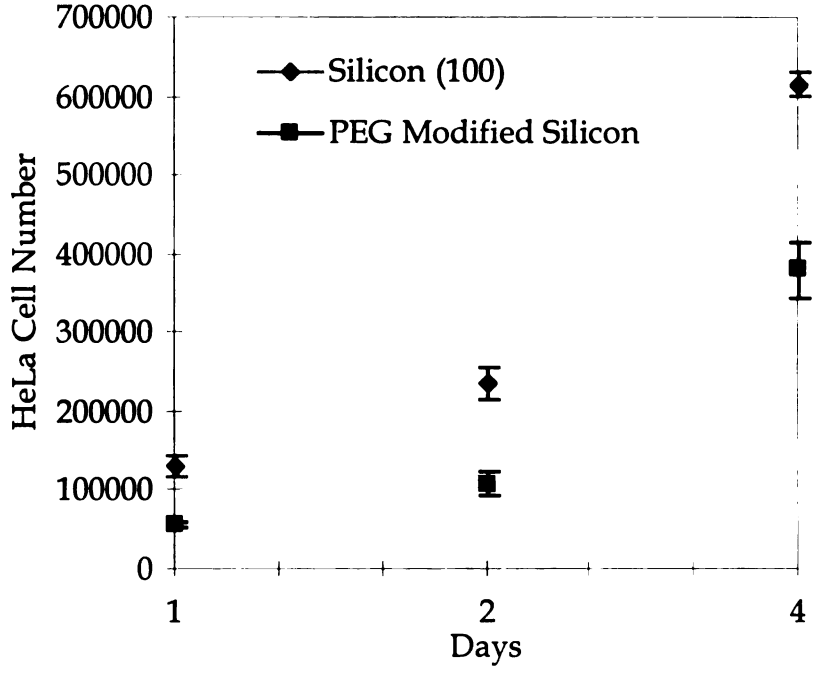


Figure 10.4.4 Adhesion of Hela cells to untreated hydrophilic silicon and PEG modified silicon.

10.4.2 Results and Discussion

Figures 10.4.1 - 10.4.4 show the adhesion of fibroblasts and HeLa cells to PEG treated and untreated silicon substrates with respect to time. Data points are mean values of three measurements with standard deviations. There was cell adhesion and a subsequent increase in the number of attached cells on both surfaces increased with time. However, significantly less initial cell adhesion was observed on PEG coated surfaces for both cell types. These observations are consistent with the results of other investigations involving cellular interaction with the PEG modified polymeric surfaces [Desai and Hubbell, 1995; Hsuie et al., 1996]. It is speculated that a decrease in the adsorption of cell adhesive proteins causes a reduction in the number of adherent cells. Furthermore, steric repulsion exerted by the PEG films together with the lack of ionic interaction between the approaching cells and PEG chains is likely to play an important role in the reduction of cell adhesion to the surface. PEG molecules in culture medium can exist in a number of spatial configurations and PEG chains can also occupy a certain volume because of the ease in rotation about single bonds constituting the polymer backbone [Harris, 1992]. Therefore, the adhesion of large numbers of cells is thermodynamically unfavorable as a result of a loss in entropy due to the reduction in the number of PEG chain spatial configurations.

Figure 10.4.2 shows the change in number of adherent fibroblasts onto differently treated surfaces, determined after 1,2 and 4 days. As seen in this graph, precoating with PEG chains caused a significant reduction in the

number of adherent fibroblasts over time. The number of cells on the silicon control samples increased with time and cell confluence was achieved in 48 hours. However, the rate of change in cell number on the PEG modified surfaces decreased with time and cell confluence had not been attained by 96 hours. The decreased rate of confluency can be attributed to the lower initial cell attachment and cell density on the modified surface. In Figure 10.4.4, the growth of HeLa cells on unmodified and modified silicon surfaces is shown for different times after seeding at a density of 10^4 cell/cm2. The numbers of adherent HeLa cells on both surfaces also increased with time, although the final cell count on modified surfaces was significantly reduced. The fact that there was some cellular adhesion on both substrates agrees with findings by Drumheller and Hubbell (1995) which showed that polymer networks with low MW PEGs (4000, 8000, and 10000 g/mol) supported confluent monolayers but with reduced confluency kinetics. The details of the interactions of cells with surfaces remain poorly understood and debatable. However, it is known that PEG chains deter or hinder the adhesion of cells to the silicon substrate and therefore can cause subsequent lower cell proliferation[Sawhney and Hubbell, 1996]. As revealed in Figures 10.4.2 and 10.4.4, both cell lines exhibited monolayer growth, but fibroblasts had a slower growth rate compared to HeLa cells, which is consistent with the results of the work of Wise et al. (1995).

The reductions in the adhesion and proliferation of fibroblast and HeLa cells increased with time and maximum value were about 55% - 62%, with the exception of the slight decrease of the reductions at the last data point for each

experiment. The reason for the observation is not clear at this time. However, it may be due to the ability of some adherent cells to reorganize adsorbed serum proteins to maximize cell interactions [Altkanov et al., 1996]. The density of specific adsorbed serum proteins on surfaces, specifically fibronectin and vitronectin, has been shown to be critical in promoting cell adhesion [Horbett and Schway, 1988]. Since, our surfaces did support some protein adsorption, this density of adsorbed cell adhesive proteins from the serum-containing culture media may have been sufficient level to support cell adhesion and subsequent proliferation.

Additionally , Norde and Lyklema (1989) reported that at low surface coverage polymer and/or protein bridging may lead to cell adhesion whereas at higher coverages, steric repulsion tends to prevent overall cell attachment. Thus, since our surfaces support lower protein adsorption, such low density polymer coverage could, in fact, support some cell adherence, although in significantly less quantities than untreated surfaces.

It is interesting to note that the rates of change of cell number on the substrates were similar despite the significantly lower initial cellular adhesion on the PEG modified surface. This seems to indicate that PEG modified surfaces do not adversely affect cell biochemical functionality and growth characteristics, but rather physically hinder cell approach and attachment to the surface. The similar rates of change of cell numbers on both surfaces suggest that cell detachment or death does not occur and that the adherent cells are growing normally on the modified substrate.

10.4.3 Conclusions

Surface Modification of silicon substrates is extremely important for potential applications in biomedical microdevices. By using functional groups such as PEG, surfaces can be tailor made to elicit specific cell and protein response as well as decrease adsorption of nonspecific biologicals. The results presented show that that it is possible to significantly suppress cellular adherance through surface modication by changing physical, chemical, and thermodynamic properties of surfaces. Furthermore, these linkage groups can be further modified by attaching biomolecules, immunomolecules, or recognition factors in order to create more biocompatible and biofunctional devices.

10.5 Influence of Material Microstructure on Cellular Proliferation

As mentioned previously, both microstructural features and surface chemistry of biomaterials can greatly affect cell adhesion and proliferation. It has been generally accepted that surfaces with high surface energy are biologically active and promote cell attachment, wheras materials with low surface energy are more likely to produce a capsule of amorphous scarlike tissue.

Studies have even showed that osteoblasts and chondrocytes can detect changes in material crystallinity. For example, it was found that chondrocytes displayed markedly different growth characteristic on highly ordered TiO2 surfaces compared to amorphous TiO2 layers [Hambleton et al., 1994].

To examine cellular response to some common microstructural elements in microfabricated materials, *in vitro* cell culture tests were conducted with the following materials: Silicon with both <111> and <110> orientations, SiO2, Poly-Silicon (unstressed, compressive, and tensile stress), Si3N4 (stoichiometric nitride), SixNy, and glass.

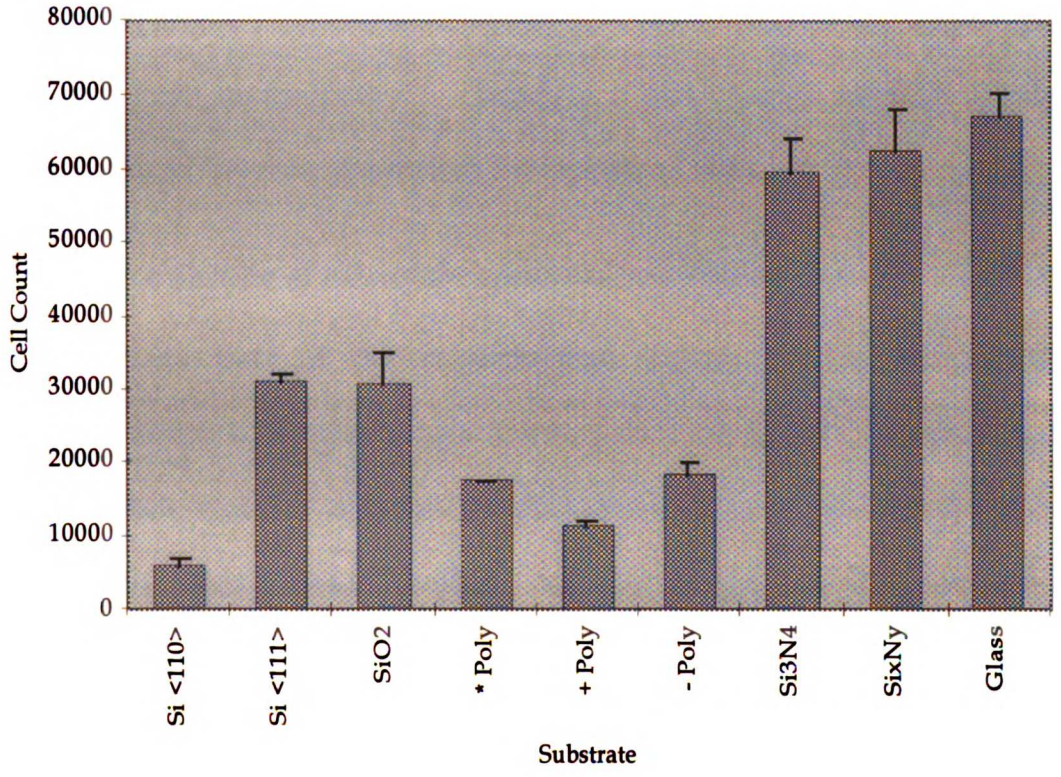


Figure 10.5.1 Cell adhesion onto substrates with different surface treatments

Although these tests were not meant to be conclusive, they further confirm the fact that cellular adherence is directly related to surface energy and chemical groups on the substrate surface (Figure 10.4.1). Both of the nitride substates had significantly greater cellular adherence and proliferation. This agrees with the fact that cells are known to display spreading and

differentiation on polylysine coated culture dishes, which present amino (-NH3) groups, better than uncoated dishes. Interestingly, all the polysilicon films displayed low cellular adherence. In fact, the regions directly adjacent the specimens were sparce with regards to cell density.

11. Islet Viability and Functionality in Microfabricated Biocapsular Environments

Previous *in vitro* studies biocapsules, using a variety of materials and fabrication techniques, have been both promising [Sun et al., 1985; O'Shea et al., 1984] and disappointing [Wu et al. 1988; Ricker et al., 1986]. Moreover, reports on in vitro glucose stimulated insulin release of comparable biocapsule designs have often been contradictory. Therefore, it was deemed essential to establish functionality of the microfabricated biocapsule *in vitro*.

11.1 In Vitro Testing of Microfabricated Culture Wafers

A large part of the experimental studies on the microfabricated biocapsule involved determining whether pancreatic islets of Langerhans could maintain their viability and functionality *in vitro* for a prolonged periods of time when encapsulated within microfabricated closed environments. Therefore, in vitro tests were designed to assess whether islets would display long term viability and retention of phenotypic expression under encapsulation conditions. Static incubation studies of encapsulated islets for extended periods of time and measurements of diffusion rates for both insulin and glucose were conducted in order to better characterized the overall effectiveness and feasibility of using microfabricated biocapsules for cell immunoisolation. Because of the configuration and dimensions of the microfabricated biocapsule, it was necessary to develop new in vitro testing protocols which would be compatible with established microfabrication and biological protocols.

The *in vitro* functionality of islets in microfabricated biocapsules under static conditions was determined by monitoring insulin secretion of islets within the biocapsules. The insulin secretion levels were compared with that of unencapsulated islets using techniques designed to simulate interstitial glucose diffusion through biocapsules. The methods used to analyze the effectiveness of our microfabricated biocapsule were modified from protocols originally developed to assess other encapsulation devices, specifically for immunoisolation of pancreatic islets of Langerhans, over the past thirty years. By utilizing these established protocols, a comparison could be made as to the overall functionality of this new biocapsule with that of traditional polymeric biocapsules.

11.1.1 Materials and Methods

Islet Isolation

The pancreatic islets of Langerhans were isolated at the Whittier Institute for Diabetes in La Jolla, California, under the supervision of Dr. Alberto Hayek and Gillian Beattie. In accordance with established animal use regulations, the islets that were used for in vitro testing of the microfabricated biocapsule were isolated from neonatal rats using a modified protocol of islet isolation originally developed by Hellerstrom et al. (1979). Briefly, the steps included removal of the pancreas from the rat, mechanical fragmentation of tissue, enzymatic digestion using collagenase, and sedimentation to separate islets, as described:

1. Place 8-9 fetal rats per scintillation vial of Type II Collagenase (11 mg Collagenase/2 ml Hank's Buffer; Sigma)
2. Decapitate five day old rats; excise pancreata and place in Hank's Buffer (pH 7.3) on ice.
3. Cut tissue into 1 mm pieces by random chopping motion with scissors in glass vial
4. Take out the Hank's; put in Collagenase/Hank's solution
5. Place vial in 37°C, 200 cycles/min shaking water bath for 5-7 minutes
6. Stop reaction with cold Hank's (10-13 ml) for 5 minutes in hood; then take Hank's out with pipette and transfer tissue into a 50 ml centrifuge vial with 50 ml Hanks's.
7. Spin for 5 minutes; remove Hank's.
8. Place 1 ml of cells in plate with 3 ml of RPMI 1640 + 10% FBS; then add additional 4 ml of RPMI.
9. Change medium every other day (the first time, only change half the medium)
10. Incubate cells at 37°C at 5% CO2 atmosphere. During culture the acinar cells degenerate and the islets remain.

Insulin Radioimmunoassay

For the quantitative assay of insulin concentrations in serum, a radioimmunoassay kit was used (Coat @ Count) . The principal is based upon competitive binding whereby ^{125}I-labeled insulin competes with insulin in the sample for sites on insulin-specific antibody immobilized to the wall of a polypropylene tube. After incubation, isolation of the antibody-bound fraction is achieved by decanting the supernatant. The tube is counted in a gamma counter where the counts are inversely related to the amount of insulin present in the sample. The insulin concentration is determined by comparing the

counts to a standard curve. The tracer has high specific activity with total counts of approximately 45,000 cpm at iodination with a maximum binding of 50%. The antiserum is highly specific for insulin, with very low crossreactivity to other proteins or compounds found in the samples. Moreover, the procedure can detect as little as 1.2 μIU/ml insulin.

Culture Wafer Fabrication

Microfabricated Silicon culture wafers were fabricated as described in Chapter 9. Microfabricated membrane pore sizes ranging from 18 nm to 3 μm were investigated. For experiments, half capsules were sterilized and placed into sterile tissue culture containers. Isolated islets were then pipetted into half-capsule wells. Each half capsule was placed in a tissue culture plate well with appropriate tissue culture media. Unencapsulated islets were also placed in culture plate wells with medium to be used as controls. All of the culture plate wells were kept under identical conditions (incubation at 37 °C, 5% CO2, in 1 ml of RPMI complete medium + 10% Fetal Bovine Serum).

Glucose stimulations

The in vitro functional capabilities of these biocapsules after various periods of static incubation were assessed using a radioimmunoassay quantifying the amount of insulin released in one multiplate well into a standard volume of medium after one-hour glucose stimulations. After overnight culture, all groups of encapsulated islets and unencapsulated islets

were washed with phosphate buffered saline (PBS) to remove insulin which might have built up during the previous culture period. After washing was completed, the concentration of insulin released at one hour was determined after both basal (1 ml of prewarmed RPMI medium containing 1.6 mM glucose) and stimulatory (16.7 mM glucose/ml RPMI medium) incubation conditions [Lacy and Kostianovsky, 1967]. Control tests using unencapsulated islets were performed under identical stimulatory conditions.

11.1.2 Data and Results

As reported in Desai et al. (1997), the viability and functionality of islets within the porous pockets of the microfabricated *culture wafer* were monitored in vitro. The culture wafer is schematically represented in Figure 11.1.1. Nutrient containing medium is allowed to diffuse only through the membrane on the bottom of the culture wafer pockets. The top of the pockets are open and exposed to the ambient environment of the incubator. After completing in vitro tests of islets within these culture wafers, the stimulus-response function of islets within *fully-assembled* biocapsules was then studied.

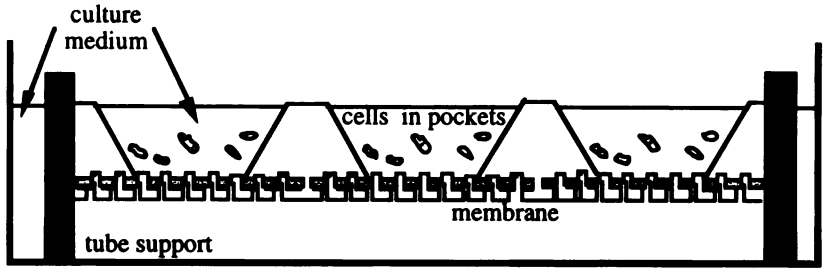


Figure 11.1.1 Schematic of cell culture wafer used to examine cell-biocapsule interactions

11.1.2.1 Viability of islets in culture wafers/ half capsules

Since the culture of pancreatic islets had never been carried out in microfabricated silicon-based environments, it was important to first determine whether these delicate primary cells could remain viable in such engineered micro- and nano-porous structures. The islets had to be carefully examined to observe any abnormalities in their growth or morphological patterns. To this end, the morphological state of pancreatic islets, placed in microfabricated culture wafer pockets, was examined daily for up to two weeks. Islets were stained with diathazone, a stain used to indicate islet cell viability. Overall, pancreatic islets in the silicon pockets and the control dishes appeared to have similar morphology. The islets were all intact and round in appearance and showed good attachment. Islets in pockets showed less dissolution or dissociation than islets in standard culture dishes. Islets in

both the culture wafer pockets and standard culture dishes exhibited slight necrosis in their central portions after 72 hours.

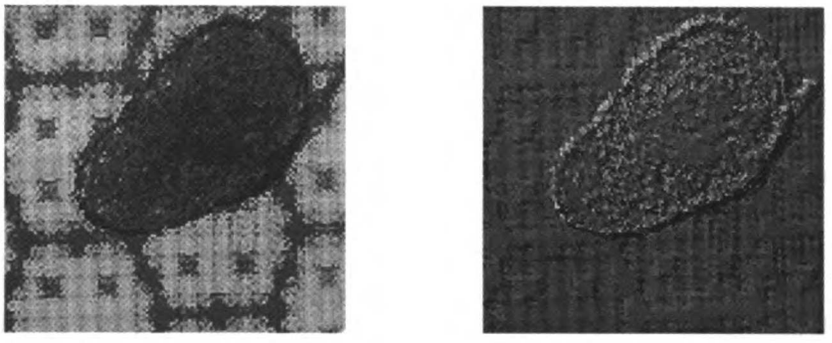


Figure 11.1.2 Pancreatic islet cultured on a microfabricated biocapsule membrane after two weeks in culture shows intact morphology.

11.1.2.2 Cell functionality and membrane permeability to glucose and insulin

The ability of pancreatic islets of Langerhans to secrete insulin in response to glucose diffusion through capsule pores was first studied in microfabricated half-capsule environments. First, islet cells were cultured on microporous membranes of 3 μm pore size. These membranes could be fabricated in a straightforward manner with standard photolithography due to their relatively large pore size. By characterizing islets on membranes with larger pore sizes, it was possible to establish whether or not the membrane architecture itself was interfering with islet viability and whether nutrients could indeed diffuse through microfabricated membranes.

Islet cells were then cultured in the pockets of silicon culture wafers with pore sizes of 78 nm, as well as in standard culture dishes as controls. Approximately 30 islets were placed in each sample. Membranes of 78 nm pore size were used for the initial rounds of testing since according to several studies, it was found that pore sizes of less than 1 μm were effective in immunoisolation membranes. Moreover, this dimension could easily be modified and optimized later, depending on the outcome of functionality studies. Glucose-supplemented medium was allowed to diffuse to the islets, from underneath the membrane, to stimulate insulin production. The concentration of insulin, secreted by the islets through the membrane, into the surrounding medium was compared between the unencapsulated islets and the islets in 78 nm pore-sized half culture wafers. The insulin concentration for half culture wafers was comparable to the unencapsulated islets, suggesting that glucose was able to sufficiently pass through the pores of the wafer pockets to stimulate islets for insulin production. It appears that the environment of the silicon pockets does not impede islet functionality and insulin secretion, as

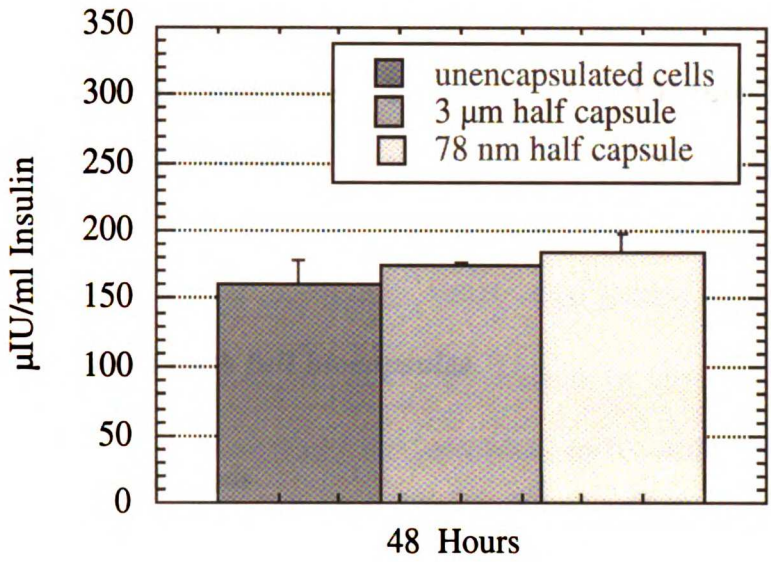


Figure 11.1.3 Insulin secretion for control and half-encapsulated islets at 48 hours (30 islets/half capsule).

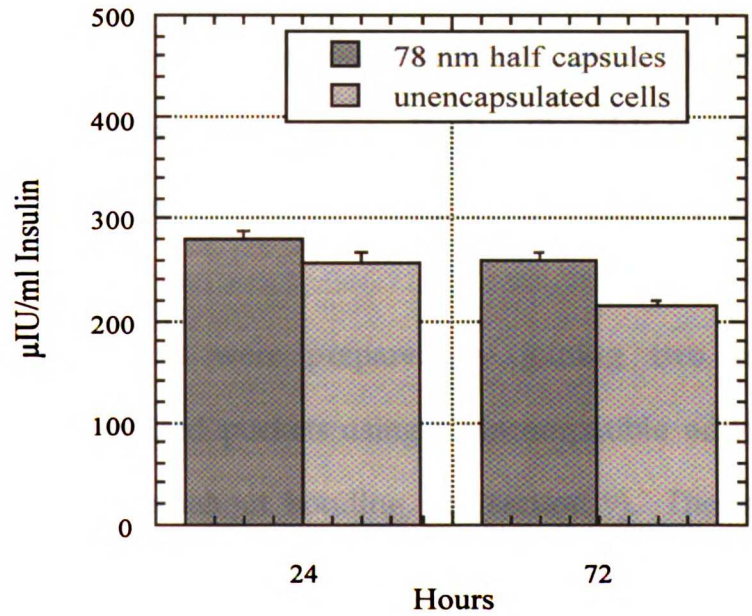


Figure 11.1.4 Insulin secretion for control and half-encapsulated islets at 24 and 72 hours (50 islets/half capsule).

compared to unencapsulated islets. Figure 11.1.3 and Figure 11.1.4 shows the secretion of insulin for control islets and islets half-half capsules from 24 to 72 hours.

11.2 In Vitro Studies with full biocapsules

11.2.1 Materials and Methods

Cell functionality studies were then conducted with islets within fully assembled microfabricated biocapsules [Desai et al., 1998]. The biocapsule structure was subsequently modified to improve diffusion parameters. Pore configuration was altered to minimize path length and membrane thickness and therefore, increase diffusion rates. The configuration of the biocapsule was changed from a L-shaped pore configuration to a straight through pore configuration (Design M2 rather than Design II). This facilitated transport of the biomolecules and seemed to improved overall performance of the biocapsules.

Full biocapsules were prepared by joining two half-capsule units containing islet-prefilled pockets using a biocompatible adhesive to form the full capsule (for more about bonding, see section 9). The insulin release by encapsulated islets was evaluated by adding stimulatory medium to wells containing islet-filled biocapsules. Immediately after adding the solutions (time zero), aliquots of medium were collected from each of the wells. Samples were collected at 10 minute intervals, while minimizing the sampling time

during which biocapsules were out of the incubator. This was of importance since insulin secretion in response to glucose is reduced at room temperature. Insulin secretion over 90 minutes was calculated by taking into account the final incubation volume and the insulin removed in the four successive samplings [Chicheportiche and Reach, 1988]. The ability of islets to return to baseline insulin secretion levels upon incubation in basal glucose conditions was also monitored. The collected samples were stored at -20 °C until radioimmunnoassayed, as described above. Mean values ± SD of the amount of insulin were calculated for each group at each time point.

11.2.2 Results

Results indicated that the secretory response of the biocapsules was similar to that of unencapsulated islets for both 3 micron and 78 nm pore sized membranes, with insulin release occurring within ten minutes of stimulation (Figure **11.2.1**). Figure 11.2.2 shows the typical insulin release profile in response to both basal (1.6 mM) and stimulatory (16.7 mM) glucose medium over 1 hour under static incubation for 78 nm pore sized biocapsules. This profile indicated that insulin and glucose diffusion occurred at sufficiently high rates through the microfabricated membrane and further validated the choice of pore density and pore path length for the microfabricated membrane. By contrast with conventional polymer biocapsules, these parameters are readily changeable and quite easily modified by changes in the fabrication process parameters.

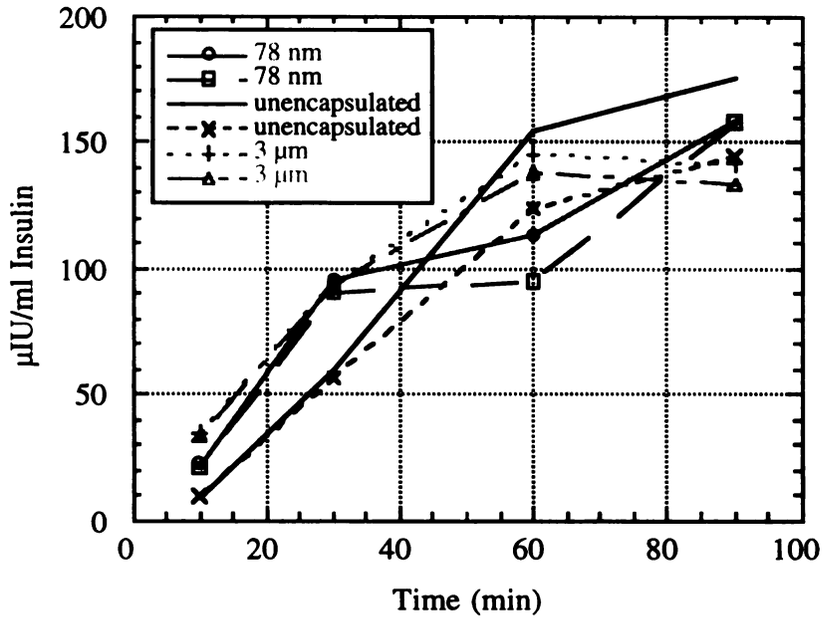


Figure 11.2.1 Insulin secretory response due to acute glucose stimulus (16.7 mM) for different pore sized biocapsules versus unencapsulated islets (20 islets/sample, n=5)

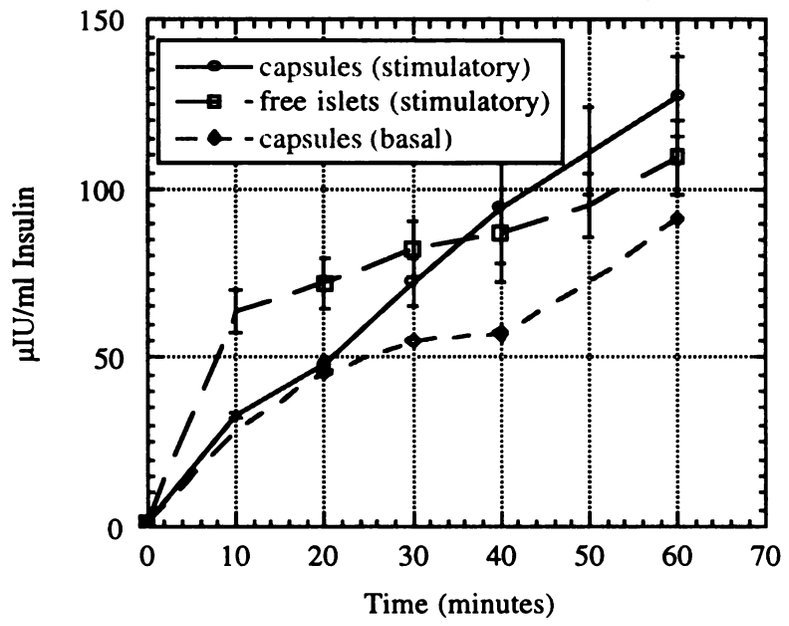


Figure 11.2.2 Basal (1.6 mM glucose) and stimulatory (16.7 mM glucose) insulin secretory response for 78 nm pore sized biocapsules versus unencapsulated islets (20 islets/capsule, n=5)

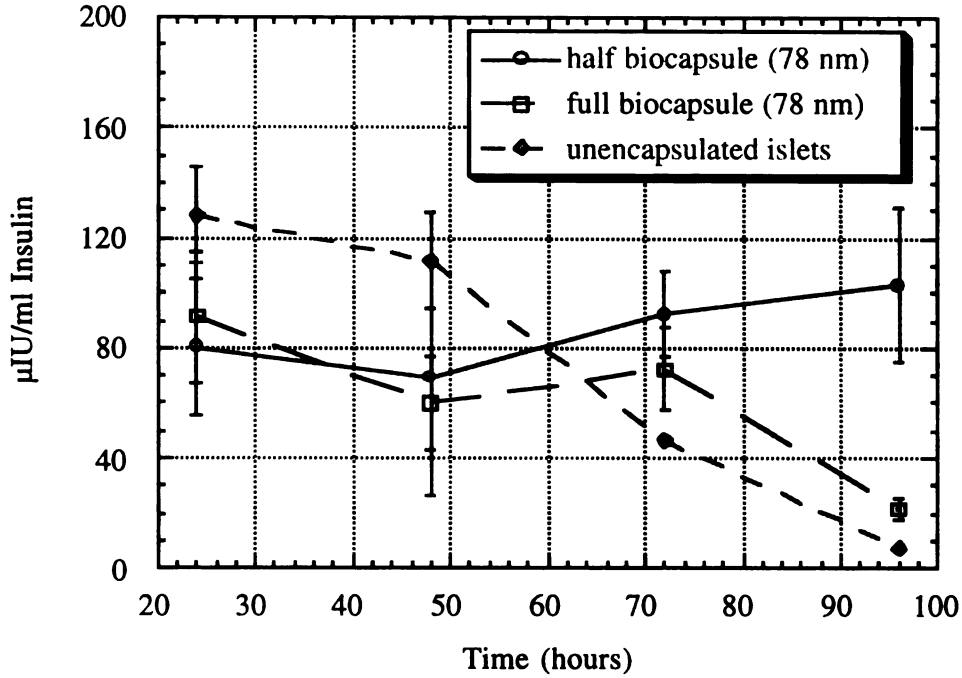


Figure 11.2.3 Comparison of insulin release from islets in whole and half biocapsules and unencapsulated islets over time in response to 16.7 mM glucose stimulus (n=5).

The next set of experiments examined the functionality and viability of islet clusters within the microfabricated biocapsule over longer periods of time. Initially, acute studies were performed monitoring islet function over 24 to 72 hours. These initial acute studies of islets within microfabricated environments were essential in determining the feasibility of using such devices as long term encapsulation vehicles. Although 72 hours is not a long period of time in terms of therapeutic applications, it is, nonetheless, sufficient to evaluate the presence of any acute cytotoxic effects of the device and/or whether the islets were receiving sufficient nutrients and support in their microfabricated environments. By comparing the encapsulated islets to islets cultured in standard biocompatible petri dishes with no diffusion barriers, it was possible to see if the encapsulated islets were being adversely affected by their encapsulation within the microfabricated biocapsule.

When insulin secretory studies were conducted comparing half and full biocapsules, it was discovered that the islets in the half biocapsules (i.e. top half of capsule open and exposed to air) had a much greater insulin release in response to stimulation than both closed capsules and unencapsulated islets (Figure 11.2.3). The reason for this was hypothesized to be that islets in half capsules had easier access to oxygen than both the fully enclosed islets and the islet completely submerged in medium. Thus, these islets retained viability and functionality much better than the other islets. Because islets require

sufficient oxygen to maintain physiologic secretory performance, it was important to address ways in which to get adequate diffusion of oxygen to the fully encapsulated islets.

Interestingly enough, the closed biocapsules, performed as well or better than free floating, unencapsulated islets, suggesting that the encapsulation did not harm the islets. Previous studies have shown that islets in culture generally develop necrotic cores after a couple days due to lack of oxygen diffusion to the central portions of the islets. Encapsulated islets, as examined post experiment, displayed similar amounts of necrosis to their central regions. The amount of necrosis, however, was not increased in encapsulated islets. In fact, islets in microfabricated structures appeared to hold their morphology much better than those not encapsulated. Furthermore, the 78 nm pore size did not hinder the diffusion of essential metabolic nutrients, as displayed by the similar insulin secretory responses of fully encapsulated and unencapsulated islets (Figure 11.2.4 and Figure 11.2.5).

As Figure 11.2.6 also indicates, the biocapsules provide a stable functional environment for the islets. Insulin secretion from encapsulated islets was maintained at similar levels throughout the studied period whereas secretory function in unencapsulated islets began to diminish rapidly. As seen in Figure 11.2.7 , fully encapsulated islets remained viable and functional as long as the unencapsulated control islets. Because rat pancreatic islets of Langerhans are primary mammalian cells, they have a finite lifespan in vitro, typically less than six weeks. The biocapsule studies, therefore, indicated that

the microfabricated environment is not detrimental to the islets and may actually prolong lifespan in vitro.

Morphological examination of the islets in opened biocapsules indicated well-defined islet architecture with little central necrosis, and no peripheral necrosis after four weeks. Additionally, there was islet attachment to the inorganic substrate. In contrast, the unencapsulated islets did not have intact outlines and displayed significant dissociation, with patches of isolated cells around the periphery.

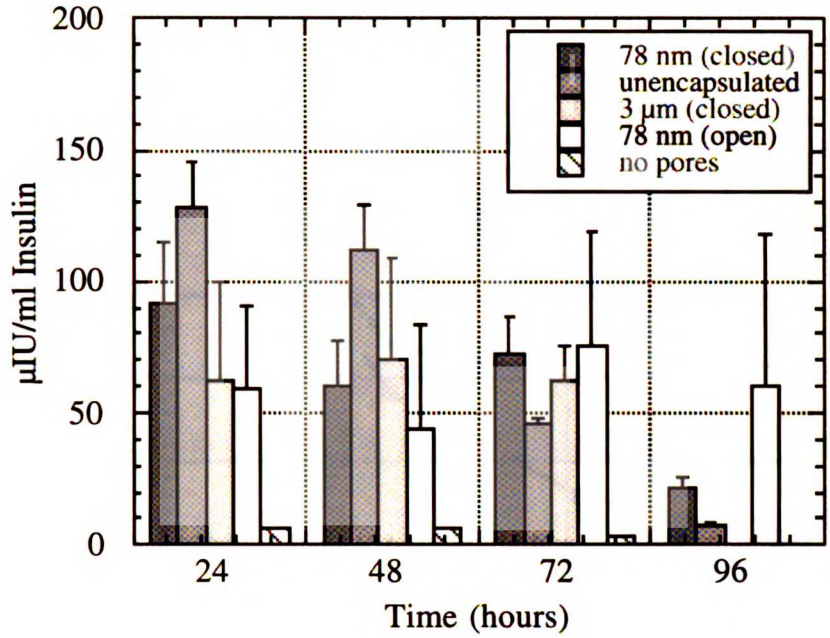


Figure 11.2.4 Comparison of insulin release of islets in different pore-sized biocapsules versus unencapsulated islets.

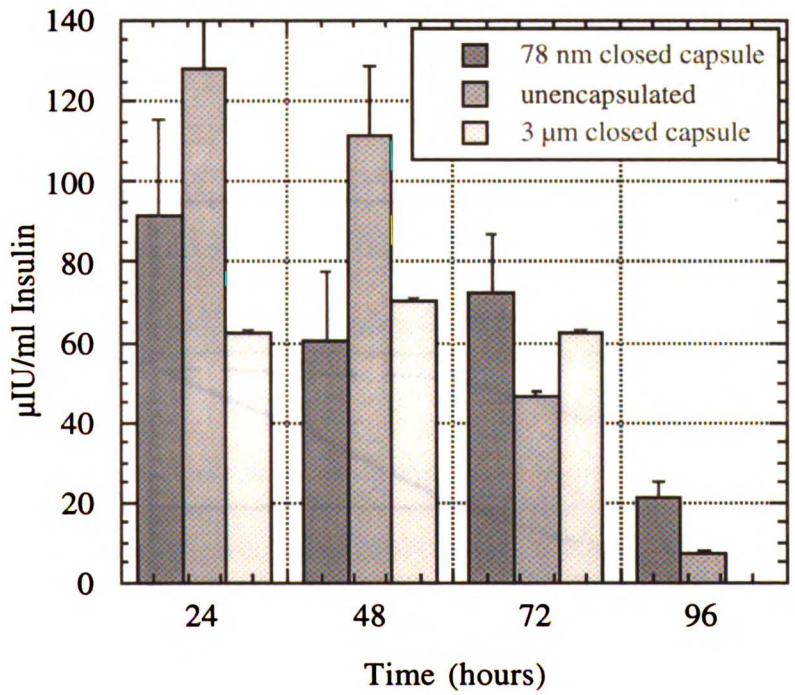


Figure 11.2.5 Biocapsules with 3 µm and 78 nm membrane pores sizes maintained greater islet functionality over several days

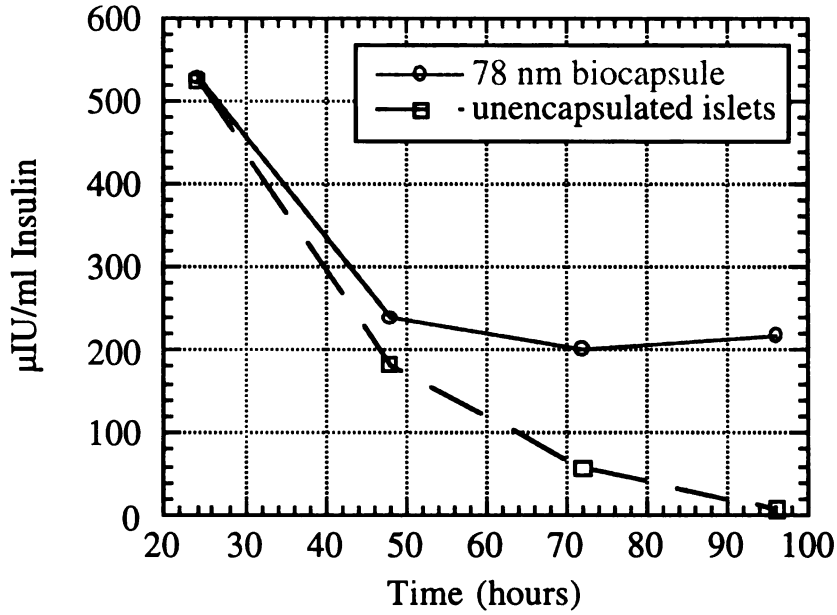


Figure 11.2.6 Insulin release for encapsulated and unencapsulated islets over time in response to a 16.7 mM glucose stimulus (n=5). The encapsulated islets maintain stability over time.

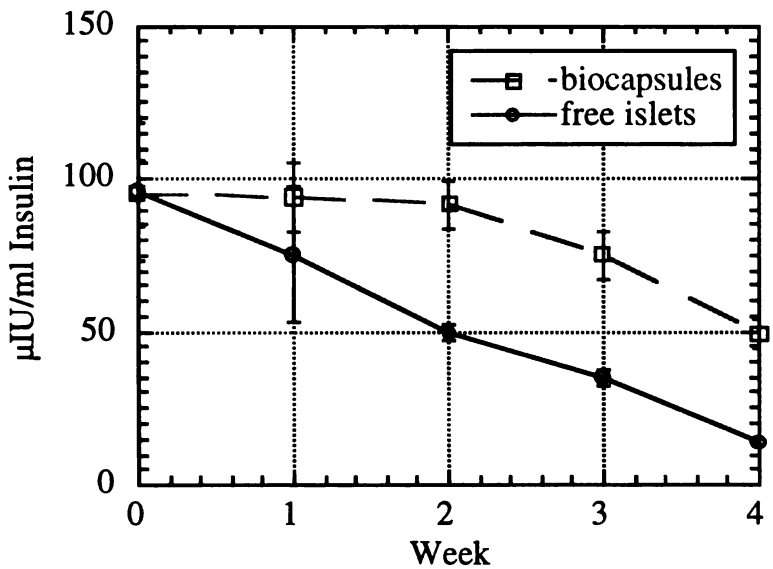


Figure 11.2.7 Insulin release for encapsulated and unencapsulated islets over four weeks in response to a 16.7 mM glucose stimulus (n=6).

Although several factors account for maintenance of islet cell viability and functionality, adhesion to an appropriate matrix might be necessary for long term survival. In vivo, normal islets cells are surrounded by the three dimensional matrix arrangement and microarchitechture of the pancreas. This structural environment not only promotes adhesion and intercellular communication but also may play a role in islet cell neogenesis [Hellerstrom and Swenne, 1991]. Islet that are maintained free floating in culture lack such a matrix and are therefore, at a disadvantage in term of long term attachment and proliferation. Therefore, because the biocapsules can provide a three-dimensional matrix environment for the cells, it seems reasonable that the islet cells would maintain greater functionality and viability within the capsule over longer periods of time [Brendel et al., 1994].

11.3 Conclusions

From the range of in vitro tests that were carried out, several conclusions can be drawn. First and foremost, the microfabricated biocapsule was shown to be sufficiently biocompatible and nonbiodegradable for the intended purposes. This was an important finding not only for the application of the biocapsule but for all microfabricated devices for biomedical applications using silicon-based components. Secondly, the biocapsule three dimensional environment could provide sufficient diffusion of nutrients, oxygen, glucose, and insulin for islet cell survival and longevity. Again, the novelty of this technology lies in the ability to optimize biocapsule pore size, path length, and capsule configuration. Thus depending on the seeding density of the encapsulated cells, biocapsule

membranes could be designed specifically to maintain adequate diffusion of essential biomolecules. Studies not only indicated that microfabricated porous silicon environments, providing partial and full containment of islets of Langerhans, maintained viability of islets but also that the functionality of these islets was preserved. Thus, the microfabricated biocapsule presents itself as a promising technology for cell transplant therapy.

12. Immunoprotection Studies of Microfabricated Biocapsules

12.1 Microfabricated Membrane Diffusion Studies

There is much experimental variability, both *in vitro* and *in vivo*, as to the exact transport properties of currently used immunoisolation membranes. Laboratories have poorly characterized their membranes in terms of specific large and small molecule diffusion properties, instead focusing on absolute performance criteria. Indeed, it is often complicated to characterize the diffusive transport properties of immunoisolation membranes due to the variability and asymmetry of membranes, most of which are polymeric. This nonuniformity in membrane architecture makes it difficult to define diffusion lengths and absolute molecular cut-off dimensions of the membrane. However, due to the uniformity and known geometry of *microfabricated* membranes, it is much more straightforward to determine diffusion parameters for a variety of molecules.

Typically, transport in polymeric immunoisolation membranes is characterized by the nominal molecular weight cutoff of the membrane. Although these values give an idea as to which molecules will can pass through a membrane, they are not always useful in determining the rate of passage or absolute selectivity through diffusion based immunoisolation membranes [Dionne et al., 1996]. It is often difficult to define diffusion lengths, pore sizes, and distributions in polymeric membranes. On the other hand, the silicon immunoisolation membrane has a well controlled geometry that is precisely specified allowing one to determine transport characteristic and diffusion

coefficients. By understanding the diffusion properties of a given immunoisolation membrane, optimization of passage of nutrients and hormonal product can be achieved as well as more accurate assessments of immunoprotection capabilities of the membrane.

The permeability of insulin, glucose, and IgG was studied in microfabricated biocapsules with various pore sized membranes. The semipermeability of microfabricated biocapsules, along with their stability and biocompatibility, may provide an improved encapsulation device for immunoisolation of cell xenografts for hormone therapy, i.e. production of insulin by pancreatic islets in response to changing glucose levels. Since immunoisolative devices are primarily diffusion based, it is essential to characterize the membrane diffusion properties in order to more accurately determine the degree of immunoprotection offered.

12.2 Diffusion of polystyrene beads of various dimensions

12.2.1 Materials and Methods

The ability of microfabricated biocapsule membranes to perform size-based exclusion of biomolecules was studied using FITC-labeled polystyrene beads of 44 and 100 nm. A suspension of beads was placed into a half-biocapsule well (2 μl) and subsequently sealed with another biocapsule half. At intervals of 24 hours, the medium surrounding the biocapsules were collected and bead concentration was measured using a spectrofluorimeter.

12.2.2 Results

It was found that biocapsules membranes of 18 nm pore size completely blocked the diffusion of 44 and 100 nm diameter polystyrene beads, while 66 nm pore sized membranes only blocked 100 nm diameter beads. No fluorescent signal above baseline was detected in the incubation medium surrounding 18 nm biocapsules after 1 and 4 days. This suggests that the biocapsule achieved absolute retention of the beads.

12.3 Permeation of IgG Through Microfabricated Biocapsule Membranes.

12.3.1 Materials and Methods

The ability of the membrane to allow diffusion of glucose and insulin while restricting the passage of IgG was studied in vitro by measuring relative concentrations using the actual biocapsule and a mini diffusion chamber constructed around the biocapsule membrane[Kessler et al., 1991]. The diffusion chamber was designed and fabricated out of acrylic. It consisted of two compartments A and B with fixed volumes of 2 ml, separated by the biocapsule membrane, sealed with o-rings, and finally screwed together (Figure 12.3.1). Nutrient and immune molecules of interest could be measured on either side of the membrane to determine membrane permeability[Burczak K. et al., 1994]. At time zero, glucose or insulin was added to compartment A and aliquots of the compartment B medium were taken at various time points. Glucose concentrations were determined by the method of Huggett and Nixon (1957) and insulin concentration was measured by radioimmunoassay. In addition,

immune molecules of interest could be measured on either side of the membrane to determine membrane immunoisolative properties.

Permeability of IgG through the microfabricated biocapsule membrane was examined using FITC-labeled IgG. This marker was placed on one side of the diffusion chamber and diffusion through the membrane into the other chamber was monitored. Membranes of 18, 66, and 78 nm pore size were tested. The diffused FITC-IgG could be detected by using a spectrofluorimeter (excitation wavelength 495 nm, emission wavelength 525 nm). The concentration of IgG was monitored daily for four days.

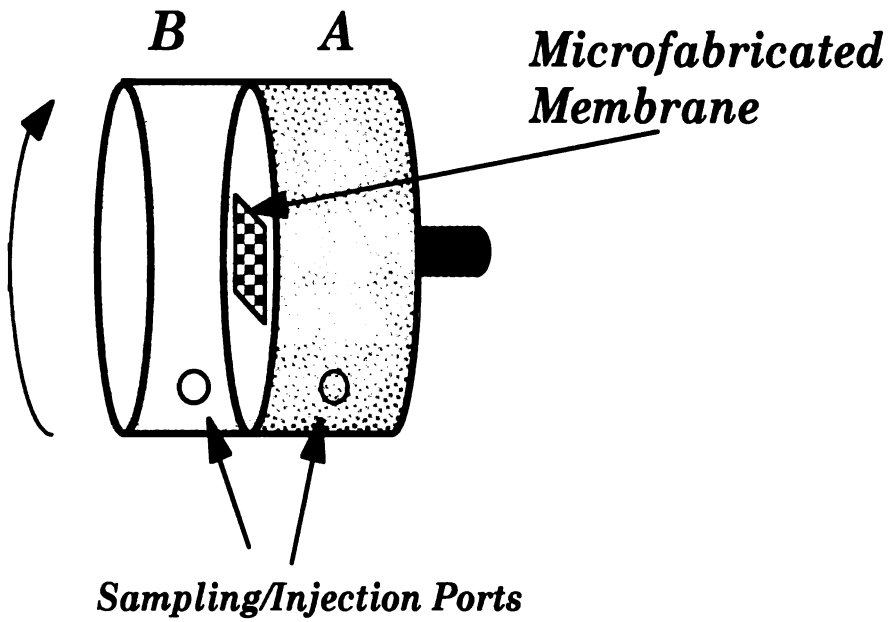


Figure 12.3.1 Experimental two compartment diffusion chamber

Additionally, IgG diffusion was measured directly from full biocapsules. The molecule of interest was pipetted into one half biocapsule wells and two half capsule were bonded together to form a full biocapsule. Biocapsules with

three membrane pore sizes were examined: 18 nm and 66 nm. At time zero, FITC labeled IgG was added to each biocapsule well (2 µl) and sealed. At various time intervals, an aliquot of the medium surrounding each biocapsule was taken. Diffusion of IgG out of fully assemble biocapsule into surrounding culture medium was monitored. At various time intervals, an aliquot of the medium surrounding each biocapsule was taken. FITC labeled IgG concentrations were also determined using a spectrofluorimeter (excitation wavelength 495 nm, emission wavelength 525 nm) and diffusion was monitored over a period of four days[Dionne et al., 1996].

12.3.2 Data and Results

The data indicated that microfabricated biocapsule membranes retarded significant IgG passage but did not completely prevent IgG permeation. As Figure 12.3.2 shows, although membranes with 18 nm pore size had a great immunoisolatory effect, both 66 nm and 78 nm pore-sized membranes allowed significant quantities of IgG through over time. The percent of IgG diffusion was less than 2% after over 150 hours through the 18 nm membranes. Compared to commonly used polymeric membranes, this rate was several times smaller indicating superior immunoprotection. For example, Dionne et al. measured an IgG concentration of 1% after 24 hours through poly(acrylonitrile-co-vinyl chloride) membranes.

Comparing diffusion rates through the 18 and 66 nm pore-sized *full* biocapsules, the data indicated that both microfabricated biocapsule

membranes retarded significant IgG passage but did not completely prevent IgG permeation (Figure 12.3.4).

Diffusion across the membrane was described by,

$$N = \frac{AD_{eff}}{L}(C_A - C_B) \qquad (1)$$

where N is the flux, A is the cross sectional area, D is the effective diffusivity, L is the membrane thickness, and C is the chamber concentration [12,13]. It is assumed that (i) there were no significant boundary layer affects in the rotating diffusion chamber and (ii) a steady-state concentration profile was established within the membrane. By coupling this with the mass balance equation, one can find the following relationship between concentration in chamber B and time,

$$\ln\frac{\alpha C_{Ao} + C_{Bo} - C_B(1+\alpha)}{\alpha(C_{Ao} - C_{Bo})} = -\frac{1+\alpha AD_{eff}}{\alpha LV_B}t \qquad (2)$$

where a = volume fraction = 1, C_{Ao} and C_{Bo} are initial solute concentrations in chamber A and B, respectively, and V_B is the chamber volume (= 2 ml). By plotting,

$$\ln\frac{\alpha C_{Ao} + C_{Bo} - C_B(1+\alpha)}{\alpha(C_{Ao} - C_{Bo})} \text{ vs. t,}$$

$$\text{and since } D_{eff} = -\frac{(slope)(\alpha LV_B)}{(1+\alpha)A} \qquad (3)$$

the effective diffusivities of IgG for different pore-sized membranes can be determined(Table 12.3.2).

Table 12.3.2. Membrane Diffusion parameters for IgG

Pore size	Effective Area (cm^2)	Absolute Area (cm^2)	Dabs	Deff
18 nm	0.08	0.00003072	1.758E-08	6.75E-12
66 nm	0.08	0.00011264	1.598E-05	2.25E-08
78 nm	0.08	0.00013312	2.028E-05	3.375E-08

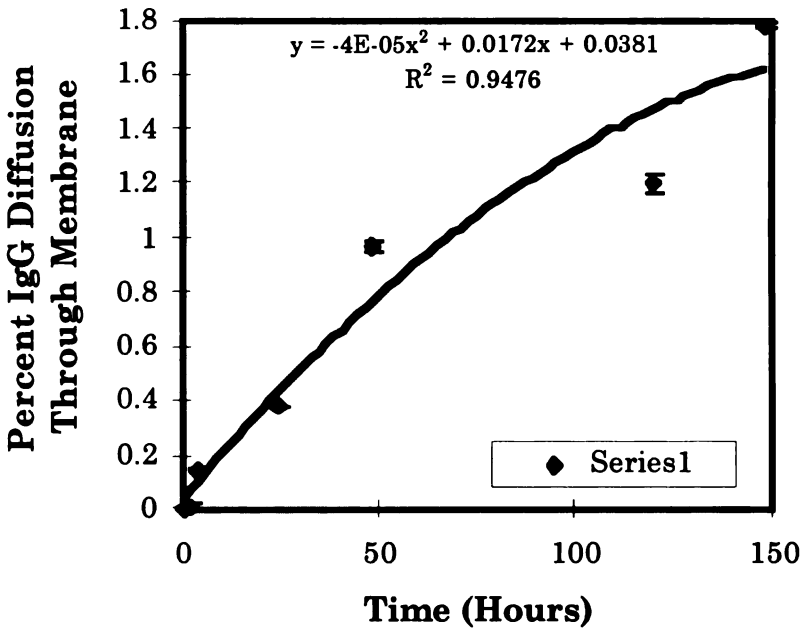


Figure 12.3.3 Diffusion of IgG through microfabricated 18 nm membrane

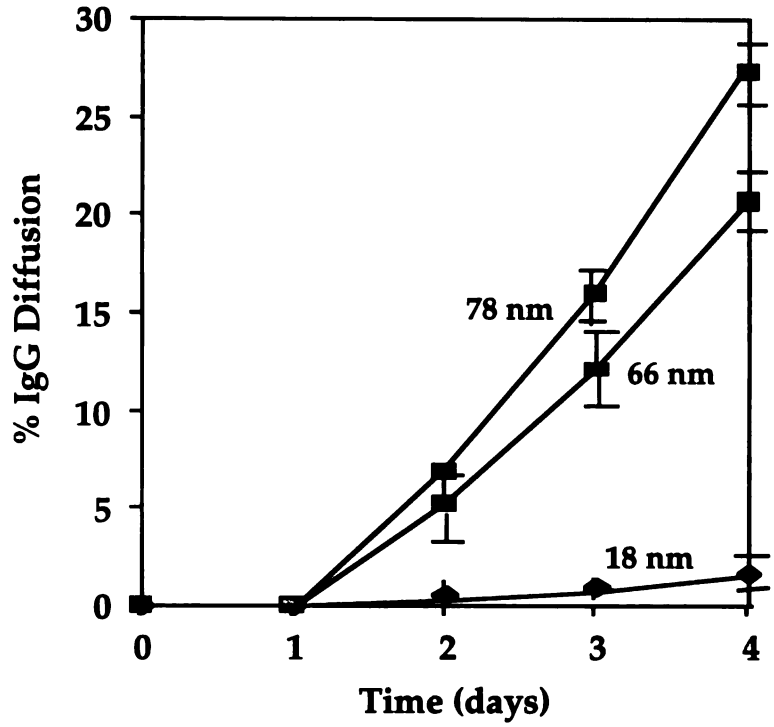


Figure 12.3.4 IgG diffusion through microfabricated biocapsules of three different pore sizes

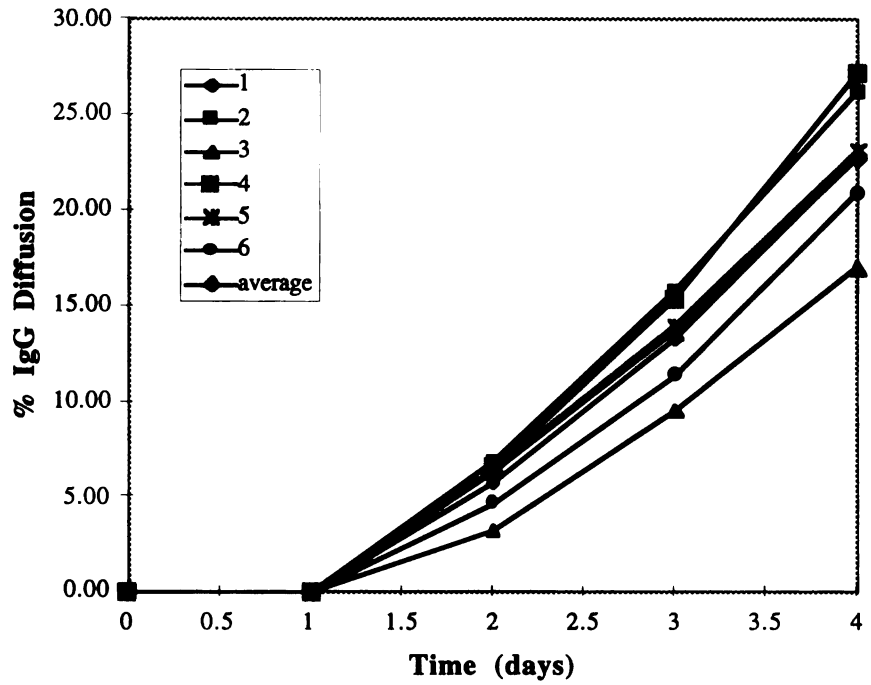


Figure 12.3.5 Diffusion profile of IgG through six 66-nm full biocapsules and average

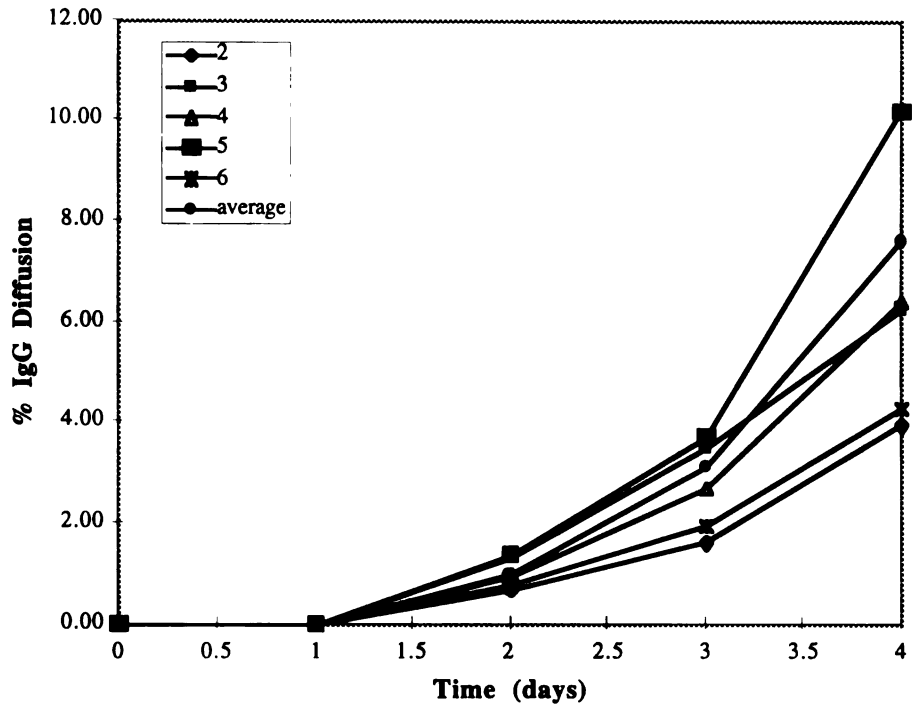


Figure 12.3.6 Diffusion profile of IgG through five 18-nm full biocapsules

12.3.3 Discussion

This study suggests that microfabricated biocapsules with membrane pore sizes less than 20 nm, may be used to immunoprotect transplanted cells. It is realized that sufficient diffusion of insulin and simultaneous blockage of IgG is not a trivial parameter in the design of a successful biocapsule membrane. Although the IgG molecule has a molecular weight of approximately 150 kD, studies have disagreed on the actual dimensions of the molecule, estimated to be somewhere in the tens of nanometers. Polymeric biocapsules usually have a broad pore size distribution, and cannot provide an absolute size or molecular weight cut-off, but rather a nominal cut-off. Also, the thicknesses of polymer

immunoisolation membranes is an order of magnitude greater than microfabricated membranes (approx. 100 μm vs. 10 μm). Therefore, microfabricated biocapsules, with uniform and well controlled pore sizes in the tens of nanometer range, may be able to provide better size based immunoisolation than conventional biocapsules.

Preliminary tests show that the passage of IgG through microfabricated membranes is greatly hindered, although not absolutely blocked. However, there are several possibilities why passage of antibodies through nanometer pore-sized membranes occurs. First, IgG diffusion may be due to small defects present in the membrane from fabrication processing. Examination of membranes post processing showed indications of small defects in some of the capsule membranes. The control and elimination of these sub-micron defects must be investigated to further improve immunoisolative properties of the biocapsule membrane. Furthermore, the bonding of the two half capsules may not have provided a complete seal and therefore, was prone to leakage of small immune molecules for the full capsule diffusion studies. Investigations into alternate bonding strategies are currently underway.

More importantly, the size, flexibility, and configuration of the IgG antibody may allow changes in conformation which permit its slow diffusion through pores greater than 18 nm, despite its molecular diameter. Therefore, changing the pore geometry of the biocapsule may be able to provide a better barrier to IgG diffusion. Since the IgG molecule is not spherical, it is likely that membrane with pore sizes as small as 90 Angstrom may not even

completely block its passage [Nakamura H, 1981]. However, using pore sizes in such small dimensions significantly decreases glucose and insulin diffusion as well.

According to some results, even alginate microcapsules that are IgG permeable and have membrane molecular weight cutoffs in excess of 70 kD (≈20 nm) can accept islet xenografts [Lanza RP, Kuhtreiber W, et al., 1995]. Therefore, it seems to be a relative property of how long these microcapsules can maintain the immunoisolation effect, rather than an absolute characteristic.

12.4 In Vitro Immunoprotection Studies Using Cells

To determine whether microfabricated biocapsules could provide immunoprotection (i.e. block the passage of key components of the immune system) specifically to pancreatic islets, the functionality of both encapsulated and unencapsulated islets in presence of serum complement and antibody was studied [Desai, Chung, et al., 1997] using a tissue culture method [Iwata et al, 1995].

12.4.1 Materials and method

Isolation of cells

Pancreatic islets cells were isolated from neonatal rats by standard methods using a modified version of the protocol by Hellerstrom et al. as described previously. Briefly, the steps included removal of the pancreas, mechanical fragmentation of tissue, enzymatic digestion using collagenase,

and sedimentation to separate islets. Islets were cultured several days before use.

Evaluation of Islet-filled Microfabricated Biocapsule Immunoisolative Properties

The survival of pancreatic islets was used to monitor immunoprotective properties of islet filled biocapsules. Biocapsules with membranes of 78, 66, and 18 nm pore sizes were studied. For experiments, biocapsule halves were sterilized and placed into sterile tissue culture containers. Microfabricated capsules were filled with a suspension of islets and assembled as described earlier. Free islets and islet filled biocapsules (10 islets per capsule) were incubated in RPMI medium with anti-rat rabbit serum and rabbit complement. at 37 °C [Sigma]. Unencapsulated islets were incubated under the same conditions. Islet viability and functionality was determined by measuring insulin concentrations in the supernatant fluid after stationary glucose stimulations (16.7 mM). The presence of insulin in solution was determined using a gamma counter. The ability of the islets in the biocapsule to maintain responsiveness to glucose stimulation in the presence of anti-rat islet antibodies and complement was compared to that of unencapsulated islets.

12.4.2 Results

As shown in Figure 12.4., the biocapsules provided significant immunoprotection to those islets encapsulated within its semi-permeable membrane. After one week in culture with antibodies and serum complement, the islets in the microfabricated biocapsules maintained original insulin secretory capacity, in contrast to the unencapsulated islets.

To validate these results, studies were extended to two weeks. As shown in Figure 12.4.2, islets in microfabricated biocapsules maintained glucose stimulated insulin secretory capability even after two weeks in the presence of antibodies and complement. Islets immunoprotected by 18 nm pore-sized membrane maintained their functionality better than those in 78 nm pore sized biocapsules, confirming that greater immunoprotectiveness was offered by 18 nm membranes. In contrast, there was a marked decrease in baseline and stimulated response in free islets. This suggests that microfabricated biocapsules may be used to immunoprotect transplanted cells. It is realized that sufficient diffusion of insulin and simultaneous blockage of IgG is not a trivial parameter in the design of a successful biocapsule membrane. Although the IgG molecule has a molecular weight of approximately 150 kD, studies have disagreed on the actual dimensions of the molecule, estimated to be somewhere in the tens of nanometers. Polymeric biocapsules usually have a broad pore size distribution, and cannot provide an absolute size or molecular weight cut-off, but rather a nominal cut-off. Therefore, microfabricated biocapsules, with uniform and well controlled pore sizes in the tens of nanometer range, may be able to provide better size based immunoisolation than conventional biocapsules.

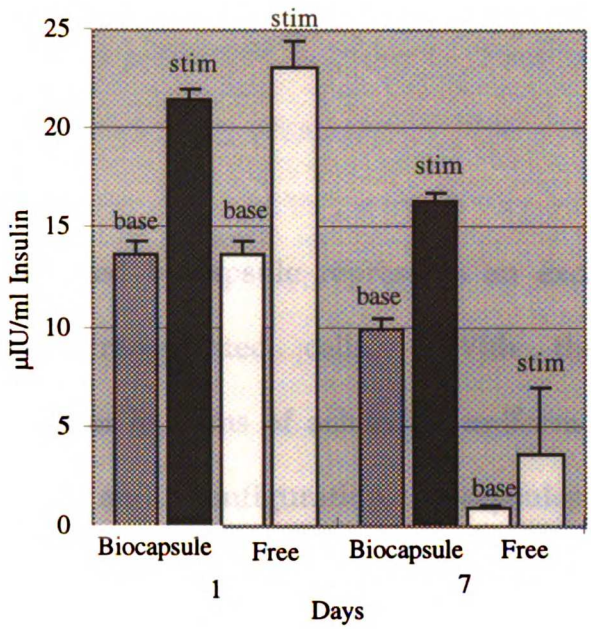


Figure 12.4.1 Insulin secretion of encapsulated and unencapsulated islets incubated with serum complement and antibody solution (20 islets/biocapsule, n=6).

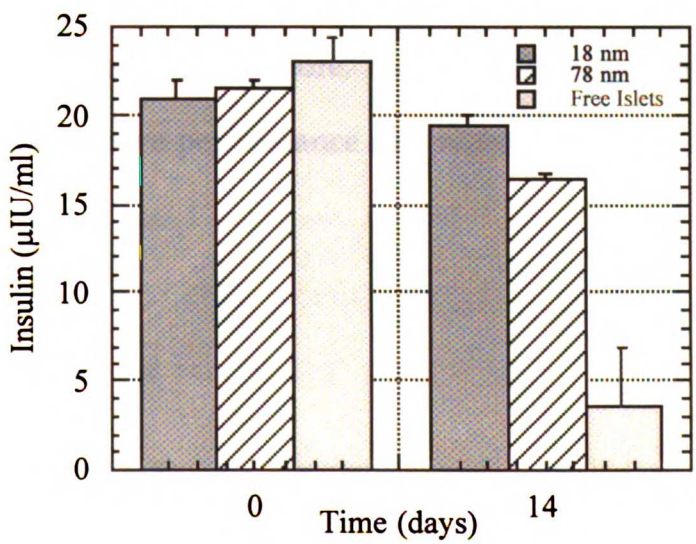


Figure 12.4.2 Insulin secretion of islets within different pore-sized biocapsules and unencapsulated incubated for two weeks with serum complement/antibody solution (20 islets/biocapsule, n=6).

12.5 *Conclusion*

The microfabricated biocapsule represents an exciting alternative for immunoisolation of transplanted cells. With the advantages that microfabrication can offer in terms of achieving uniform and well controlled pore diameter, length, and configuration, biocapsules can potentially be engineered and optimized precisely to one's desired specifications. This is especially important in developing a size-based immunoisolation device for transplanted cells such as pancreatic islets of Langerhans. Current studies are underway to investigate optimization of pore size to attain both immunoisolation and adequate hormonal diffusion out of the biocapsule for therapeutic applications. Furthermore, biocapsules fabrication strategies are being modified to improve performance and reproducibility.

13. In Vivo Studies of Microfabricated Immunoisolating Biocapsules

In *vivo* testing was performed in order to examine the effectiveness of microfabricated biocapsules as an immunoprotective device for isolated allo- and xeno-geneic pancreatic islets. Furthermore, in vivo studies were conducted to test the capacity of the encapsulated cells to survive after implantation into a foreign host. Discordant species were chosen as hosts animals in order to assess the protection of the cells from a xenogeneic immune response. From these small animal studies, important knowledge was gained regarding the functionality of the biocapsules, as well as a better understanding of the immune response to both encapsulated islets and microfabricated devices and materials.

13.1 The microfabricated Biocapsule in vivo: implantation of islet-filled biocapsules in normoglycemic mice

These preliminary experiments were performed in order to assess the biocompatibility of the islet- filled microfabricated biocapsules and the survival of the transplanted recipient animal. Studies were performed in collaboration with the Whittier Institute for Islet Research in La Jolla, CA [Desai, Hayek, Beattie, and Ferrari, 1997 *unpublished*].

13.1.1 Materials and Methods

Isolation of Neonatal Rat Islets

Five day old Sprague-Dawley rats were sacrificed by decapitation. Pancreata were removed and collected in ice cold Hank's Balanced Salt Solution

(HBSS) containing 1% Bovine Serum Albumin (BSA). Subsequently, the pancreata were minced with dissecting scissors and digested with Collagenase Type V (1 mg/ml). The digested tissue was resuspended in RPMI 1640 medium containing 11.1 mM Glucose supplemented with 10% Fetal Calf Serum, 2 mM glutamine, 50 IU Penicillin and 50 μg/ml Streptomycin, and seeded in 6 multiwell plates for culture at 37°C in an atmosphere of 95% humidified air/5 % carbon dioxide to allow for cell attachment. The cultures were maintained for five days with daily changes of culture medium. At the end of the period, the islet cultures were devoid of non-endocrine tissue and could be transferred to the microfabricated biocapsule. Approximately 50 islets were pipetted into each half biocapsule and the half was then sealed with an empty half biocapsule.

Biocapsule Implantation

Four out of five Non-Obese Diabetic mice, 16 weeks old, without any signs of overt diabetes, underwent median laparotomy for the implantation of microfabricated biocapsules in the peritoneal cavity. One biocapsule was inserted into each animal. Blood glucose levels were evaluated before the biocapsule implantation and once a week thereafter for the following thirty days. At the end of thirty days, the mice were sacrificed and laparotomized for evaluation of the tissue surrounding the implants. Examination of retrieved biocapsules was also performed.

13.1.2 Data and Results

This was the first attempt to implant microfabricated biocapsules in animals. Therefore, several issues regarding biocapsule assembly procedures, surgical protocols, and biocapsule/animal monitoring had to be sorted out. Microfabricated biocapsules were implanted in non-obese diabetic (NOD) mice without any overt signs of diabetes. NOD mice typically become spontaneously diabetic at age 35 to 36 weeks, with the incidence being more prevalent in females (80-85%) than males (35-40%). Both control and implanted mice were normoglycemic at the start of the experiment. The goal of this study was to reveal information about the biocompatibility and immune response to implanted microfabricated biocapsules.

All four mice were successfully implanted with microfabricated biocapsules in their peritoneal cavities by a small surgical incision. Of the four implanted mice, one displayed the condition of anasarca the day after the implantation but this conditions resolved spontaneously. The remaining three animals were in good physical condition during the entire experimental time period. Blood glucose levels were in the normal range both before the implantation and throughout the experimental period.

Upon examination of the laparotomized animals after thirty days, several interesting features were observed. One microfabricated biocapsule was found attached to the inferior surface of the liver. Another capsule was found close to the urinary vesicles. The remaining two capsules were proximal

to the intestinal loops. None of the tissue surrounding the implants showed any macroscopic overthrow. However, a mild fibrous tissue layer, a few cells thick, was found surrounding all of the devices. This layer was less than 100 microns in thickness. The biocapsules and the surrounding tissues were harvested for histological examination. None of the devices showed any macro- or microscopic changes in surface appearance. Again, no degradation or corrosion was observed. There was some protein and/or biomolecule adherence to the devices, but the channels were not visibly blocked.

Table 13.1.1 Glycemia Levels in NOD mice

Mouse #	Before Transplant	After Transplant			
		Wk 1	Wk 2	Wk 3	Wk 4
1	68	--	--	--	--
2	81	88	78	89	90
3	92	87	75	82	72
4	79	92	72	91	75
5	67	67	61	90	87

13.2 Immunoprotection of Encapsulated human and rat islets in STZ-mice

Microfabricated biocapsules containing rat or human islets were implanted into NIH Swiss mice, both normal and diabetic. In the diabetic animals, blood glucose was monitored daily. After 1 week animals were sacrificed, the capsules were removed and examined macroscopically. The encapsulated islets were tested in vitro for ability to respond to glucose stimulation, then cells were fixed and embedded in paraffin for histological examination.

13.2.1 Materials and Methods

Mice were rendered diabetic with Streptozotocin (200 mg/kg IP; Sigma Corp. St. Louis, MO). Only mice with 2 consecutive blood sugar values >350 mg/dl were considered diabetic. Blood was withdrawn from the tail vein for glucose measurements using a portable glucose meter (Surestep, Lifescan, Milpitas, CA).

STZ induced diabetic mice were anesthetized with pentobarbital (55 mg/kg IP). Unencapsulated islets were implanted under the kidney capsule using a positive displacement pipette. Capsules were implanted either SQ or IP through a midline incision.

After mice were sacrificed, the biocapsules were carefully removed and placed in RPMI with 1.6 mM Glucose for 30 minutes at 37^0C to allow islets to reach basal insulin release levels. Thereafter, biocapsules were incubated for 1 hour in RPMI with 1.6 mM glucose followed by 1 hour in RPMI with 16.7 mM glucose for 1 hour. Medium was frozen until assayed for insulin by RIA. Stimulation indices (SI) were calculated by comparing the stimulated to unstimulated values.

Histology was performed after biocapsule explantation . Capsules were examined both macro- and microscopically for any signs of inflammatory reaction. They were then carefully opened and the cells were immediately placed in freshly made 4% paraformaldehyde overnight. Tissue and cells were embedded in paraffin, dehydrated and 5μ sections were stained with hematoxylin and eosin.

13.2.2 Data and Results

Mouse #	Treatment	B.S. d-1 to d+7 (mg/dl)	Capsule appearance	In vitro stim (SI)	Histology
Diabetic:					
RM5	1000 human islets IP (3 caps)	366,388,276,403, 377,486,361, 450	broken	1.9	lymphocyte infiltration
RM6	500 rat islets IP (2 caps)	450,361,111,74, 116,345,210,died	broken		lymphocyte infiltration
Normal:					
RM1	200 human islets in kidney	n/a	n/a		lymphocyte infiltration
RM2	150 rat islets in kidney	n/a	n/a		lymphocyte infiltration[a]
RM3	200 human islets IP (2 caps)	n/a	1 broken 1 unbroken	1.4	lymphocyte infiltration undamaged islet, no infiltrate[b]
RM4	100 rat islets IP (2 caps)	n/a	broken	1.5	lymphocyte infiltration
RM7	empty SQ	n/a	broken		no infiltrate
RM8	empty IP 50 human islets SQ (1 cap)	n/a	broken broken	1.4	no infiltrate lymphocyte infiltration

[a] see Fig. 13.2.1 a
[b] see Fig. 13.2.1 b

13.2.3 Discussion and Conclusions

In the interpretation of these results, it was important to take into account time limitation in the design and performance of these experiments.

The surgeon who assisted had very little experience in handling the biocapsules, which may explain the high number of mechanical failures. However, it was clear that the implanted biocapsules were highly biocompatible and did not appear to be immunogenic in any way. An inflammatory reaction to the capsules themselves not observed when placed either subcutaneously or intraperitoneally. The biocapsules did have a tendency to agglutinate when placed IP, most probably a reflection of the relative size of the capsule compared to the abdominal cavity of the mouse.

The fact that the human cells in the intact capsule did not show any evidence of rejection is very encouraging, suggesting that the membrane used in the microfabricated biocapsule is an effective immunoprotectant. Functionally all encapsulated islets showed slight elevated insulin release following glucose challenge. These results suggest that given more time and adequate support, improved engineering of the biocapsules will provide a viable immunoprotectant device for cell xenotransplantation experiments.

The problems encountered by some of the initial islet xenografts was probably due to several factors. Since the transplanted islets represented only a small fraction of the islets needed to restore normoglycemia, the implanted islets were subjected to significant stress to produce adequate quantities of insulin. This eventually could have led to cell exhaustion and a decrease of glucose-stimulated insulin release capacity. Lum et al. (1992) experienced similar results in his studies with rat pancreatic islets in BALB/C mice. His study revealed a tendency for capsules to clump together and therefore, induce

islet necrosis due to insufficient diffusion of nutrients and oxygen. In contrasts, capsules removed from healthy animals displayed no clumping or cellular overgrowth and showed insulin secretory competence post explantation. The post-explanted functionality of our capsules was similar, in that those that were intact and non-agglutinated were fully functional but those that had clumped, displayed cellular infiltration and overgrowth.

It has been commonly accepted that the major limitation in the development of a clinically feasible bioartificial pancreas is related to the fibroblastic response of the implant. The fibroblastic reaction significantly reduces diffusion of major nutrients to the islets. This is even a greater problem with devices which house clusters of suspensions islets (i.e. more than one islet per capsule). Islet populations in such devices must compete with each other for oxygen and other nutrients, making rapid diffusion kinetics even more crucial. This is why Sun et al. reported that the duration of normoglycemia was significantly improved when the size of capsules was reduced from 0.7 to 0.25 mm in diameter. Our microfabricated biocapsule has a maximum diffusion distance of 0.5 mm, well within the range of previously studied capsules.

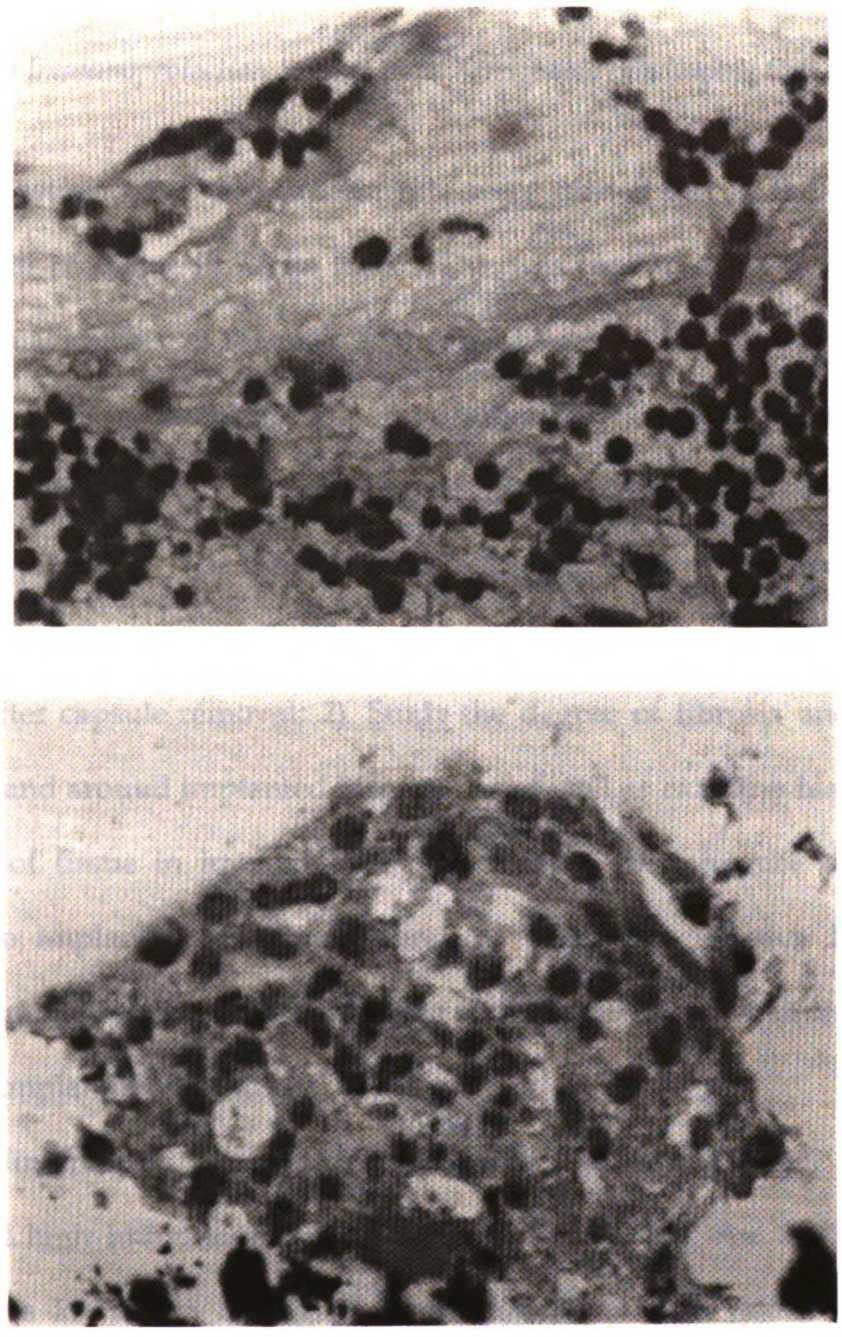


Figure 13.2.1 Micrographs of (a) unencapsulated and (b) encapsulated human islet after retrieval from rat implantation

13.3 In Vivo Immunoprotection Studies with Xenogeneic Insulinoma Cell Lines

The last set of *in vivo* experiments were carried out at the Institute of Experimental Medicine in Rome Italy. This was conducted to evaluate the *in vivo* functional efficacy of the microfabricated biocapsule containing insulinoma cells in terms of immunoisolation, biocompatibility, and glucose-stimulated insulin secretion after intraperitoneal implantation without use of immunosuppressants. The three main objectives of these experiments were to: 1) Examine cell viability in biocapsules (after one week) by performing cytology after capsule removal; 2) Study the degree of fibrosis and/or tissue damage in and around implanted biocapsules as well as examine histology and pathology of tissue in implant area; 3) Evaluate the presence of cellular adhesion to implanted biocapsules; and 4) Determine whether biocapsules prevent immune rejection by monitoring viability of highly immunogenic cell lines after implantation in mice.

Insulinoma cell lines were chosen due to their availability, stability in culture and high immunogenicity in xenogeneic species. The rat insulinoma line (RIN) is known to be highly immunogenic in mice while the mouse insulinoma cell line (βTC6F7) is a cell transplant more readily accepted, but still immunogenic, by the BALB-C mouse. It was hypothesized that both insulinoma cell lines would be rejected if inoculated intraperitoneally as free

cells in control mice, but would remain viable if immunoprotected by placement in the microfabricated biocapsule.

13.3.1 Materials and Methods

Experimental Design

Table 13.3.1 outlines the experimental set up that was followed. Two different pore sized biocapsules were evaluated, 18 and 66 nm. Two cell-filled biocapsules were implanted into each recipient mouse. Parallel in vitro experiments were run in concert with the in vivo study. Mice with no capsules and with empty biocapsules were used as controls.

Table 13.3.1 Experimental Design for in vivo insulinoma study

Site	Capsules	Cells	Capsules	
			18 nm	66 nm
3 BALB-C mice	2 (18nm)/ mouse	rat insulinoma (RIN)	6	0
1 BALB-C mouse	2 (66nm)/ mouse	RIN	0	2
3 BALB-C mice	2 (18nm)/ mouse	Mouse insulinoma(βTC6F7)	6	0
1 BALB-C mouse	2 (66nm)/ mouse	βTC6F7	0	2
3 BALB-C mice	no capsules	RIN	0	0
3 BALB-C mice	no capsules	βTC6F7	0	0
1 BALB-C mouse	2 (empty 18 nm)	none	2	0
Total mice: 15				
in vitro	4 (18 nm) + 2 (66 nm)	RIN	4	4
in vitro	4 (18 nm) + 2 (66 nm)	βTC6F7	4	4
		Total capsules:	22	12

Insulinoma Cell Lines

Two insulinoma cell lines were encapsulated within microfabricated biocapsules: a rat insulinoma(RIN) line and a mouse insulinoma (βTC6F7) line. RIN cells produce insulin under sub-physiological glucose levels and have been characterized by Knaack et al. βTC6F7 is a non-clonal cell line derived from a male mouse insulinoma arising in a lineage of RIP-Tag transgenic mice expressing an insulin-promoted SV40 T antigen hybrid oncogene in pancreatic b cells. Insulin secretion from these cells is regulated by physiological glucose concentrations, and they are equivalent to βTC7 cells, which have been described by Efrat et al.(1993). These cells maintain a stable phenotype of glucose responsiveness in the physiological range. However, at passage 18-20, they become responsive to sub-physiological glucose levels, and eventually cease to respond to glucose. F7 is a cell clone derived from βTC6 cells at passage 18 by soft-agar cloning (Knaack et al., 1994). These cells maintain a stable phenotype of glucose responsiveness in the physiological range till at lease passage 58. Cells grow as clumps on tissue culture plates. They are subcultured weekly or when about 50% confluent, by trypsinization with 0.05% trypsin, 0.5 mM EDTA, and dilution 1:3-1:5. The cells are refed twice a week.

The mouse insulinoma cell line was hypothesized to be a cell transplant more readily accepted by the BALB-C mouse when both in the biocapsules and when inoculated subcutaneously in control mice. The rat insulinoma line, thought to be highly immunogenic in mice, was hypothesized to be rejected by

the mice when injected as free cells, but remain viable if immunoprotected by the capsules.

Cell Cultures

RIN were cultured in M199 medium (Gibco-BRL, Gaithersburg, MD) supplemented with 10% FCS, 2 mM glutamine, 50 IU Penicillin, and 50 μg/ml Streptomycin at 37°C in an atmosphere of 95% humidified air and 5% CO2. βTC6-F7 cells were cultured in Dulbecco's modified Eagles Medium (DMEM) containing 11.1 mmol/l glucose supplemented with 15% horse serum, 5% FCS, 50 IU Penicillin, and 50 μg/ml Streptomycin at 37°C in an atmosphere of 95% humidified air and 5% CO2. Cells were subcultured weekly. RIN cells in culture were split 1:4 while βTC6F7 were split 1:3.

Insulinoma Cell Encapsulation

RIN and βTC cells were trypsinized (RIN: 3-4 million cells in 12 ml; βTC6F7: 3 million cells in 5 ml) and plated for 24 hours as described prior to encapsulation. Cells were adjusted to appropriate concentrations by spinning down and resuspending so that they were ready to be placed in capsules. After spinning, medium was removed to yield a 2 ml cell suspension with approximately 1.5 million cells per ml. Approximately 3 μl of cell suspension was pipetted into each capsule well. Since, the concentration of the suspension for both cell lines was approximately 1.5 million cells per ml, each capsule well contained about 4500 cells. Then half capsules were sealed with another half capsule as described previously. Sealed biocapsules were stored in appropriate

culture medium for 12 hours before implantation. Culture medium was changed for the in vitro biocapsules every other day.

Biocapsule Implantation

Microfabricated biocapsules were implanted into the peritoneal cavity of each mouse. Anesthesia was administered (Ketalar; 0.2 mg/s.c) and a small amount of ether administered by mask was used to as needed to subdue mice. A median 1.5 cm laparatomic incision was made and biocapsules were inserted in the medial right flank over the liver wall, to prevent displacement of the biocapsules among the bowel loops. Each incision was closed by suture. Free insulinoma cells (2.2×10^5) were injected intraperitoneally into mice two days later

After 8 days, mice were sacrificed and biocapsules were removed for cell viability and functionality tests. The tissue surrounding the capsules was also removed for histological evaluation.

Cell viability and functionality was evaluated by an insulin release test. Cell functionality in explanted capsules and biocapsules maintained in vitro were compared. After explantation, capsules containing RIN cells were washed twice at 37°C for 30 minutes with a glucose free buffer containing 114 mM NACl, 25.5 mM NaHCO3, 10 mM HEPES, 2.5 mM CaCl2, 4.7 mM KCl, 1.21 mM KH2PO4, 1.16 mM Mg SO4, and 0.1% BSA (pH 7.2). Thereafter, cells were incubated for 1 hour in the same buffer without glucose and then with 2.8 mM glucose. At the end of this incubation period, aliquots of the supernatant were collected and stored at -20°C for subsequent insulin radioimmunoassay.

Microfabricated biocapsules containing βTC cells were washed twice at 37°C for 30 minutes with glucose free Krebs Ringer Buffer (KRB) containing 119 nM NaCl, 4.74 mM KCl, 2.54 mM CaCl2, 1.19 mM MgSO4, 1.19 mM KH2PO4, 25 mM NaHCO3, 10 mM HEPES, and 0.1% BSA (pH 7.4). Cells were incubated successively for 1 hour in free KRB in the absence of glucose and then in KRB with 16.7 mM glucose.

Insulin concentrations were determined by the dextran-charcoal method using an anti-insulin antibody from guinea pigs. Porcine insulin was used as a standard (Sigma) and 125-I insulin was used as the hormonal radiotracer (New England Nuclear, MA).

13.3.2 Data and Results

In vitro static incubation study

Microfabricated biocapsules incubated *in vitro* for eight days showed stimulus secretion coupling of glucose and insulin for encapsulated insulinoma cells (Table 13.3.2 and Figure 13.3.1). As shown in Figure 13.3.2, the static incubation study resulted in insulin secretion from the microfabricated biocapsules containing RIN cells in response to basal glucose levels and stimulatory glucose (2.8 mmol/L) levels. The stimulatory index (SI = stimulatory/basal insulin secretion) in picograms (pg) for RIN cells in 18 nm pore-sized microfabricated biocapsules was approximately 1.7. In 66 nm pore-sized biocapsules, the basal value was 12.0±5.65 and jumped significantly to 71.3±17.25 under stimulatory conditions, an almost 6-fold increase. Similarly,

encapsulated βTC6F7 cells also displayed basal and stimulatory (16.7 mmol/L) insulin release, with a SI of 4.3 for 18 nm pore-sized biocapsules (Figure 13.3.3). And, 66 nm pore-sized biocapsules *in vitro* containing βTC6F7 cells had a SI value of 9.1.

Table 13.3.2. Insulin Secretion from Glucose Stimulated Biocapsules where SI = stimulatory index (basal insulin secretion over stimulated insulin secretion)

Cell		SI (18 nm)	SI (66 nm)
RIN	*In Vivo*	1.5 ±0.4	1.7±0.2
	In Vitro	1.9±0.05	5.9±0.9
βTC6F7	*In Vivo*	3.6±0.3	1.7±0.2
	In Vitro	4.3±0.7	9.2 ±0.5

In vivo study

After eight days of intraperitoneal implantation, glucose stimulated insulin secretion from the retrieved biocapsules was examined under static conditions. Microfabricated biocapsules were easily located in the abdominal cavity of laparotomized mice. Three biocapsules remained in the original implantation site, while the remaining biocapsules migrated to either the abdominal cavity, bowel loops, or mesentery. Nonetheless, all biocapsules seemed mechanically intact with not macroscopic changes in surface architecture or properties. Tissue surrounding the biocapsules found in their original implantation site showed either showed no abnormalities while tissue around migrated biocapsules displayed minor neutrophil infiltration. Some

capsules had agglutinated during their migration but were mechanically intact nonetheless.

In vivo, the βTC6F7 cells remained viable in microfabricated environments. The stimulatory index for 18 nm and 66 nm pore size microfabricated biocapsules was 3.6 and 1.75, respectively (Figure 13.3.4). The xenogeneic RIN cells encapsulated in 18 nm biocapsules were also able to maintain their functionality and displayed basal and stimulatory insulin secretion of 2.23±1.12 and 3.10±0.5, corresponding to a stimulatory index of approximately 1.5 (Figure 13.3.5). In 66 nm pore sized biocapsules, RIN cells had a stimulatory index of approximately 1.7. In contrast, the freely injected RIN and βTC6F7 cells were undetectable in the peritoneum as well as in any other organ (liver, kidney, lungs, or brain) as determined by careful autopsy (Figure 13.3.6). The peritoneum is known to elicit a strong immune reaction to these free insulinoma cells. Viable free insulinoma cells are expected to form a tumor nodule. However, it was nonetheless observed that all $2x10^5$ free insulinoma cells were completely taken up by the immune system while encapsulated insulinoma cells survived and maintained functionality.

13.3.3 Discussion

Several issues regarding microfabricated biocapsule implantation and effectiveness were revealed in these experiments. Insulinoma cells did maintain their viability within microfabricated biocapsules over the studied period. However, it was shown that encapsulated βTC6F7 cells *in vitro*

maintained a greater stimulatory insulin response than *in vivo*. Also, the size of the pores of the biocapsule greatly affected the secretory response. The lower insulin response in the 66 nm biocapsules implanted compared to the implanted 18 nm biocapsules could be attributable to the penetration of immune molecules through the relatively large membrane pores and subsequent attack of encapsulated cells. The size of antibody and complement components of the immune system has been hypothesized to be between 20 and 50 nanometers [Zhang, 1997]. Previous studies on biocapsules have suggested that pore sizes smaller than 100 nm are effective in hindering passage but still do not completely block immune molecule from reaching the target cell [Iwata et al., 1995]. Therefore, it is likely that a pore size of 66 nm still allows passage of immune molecules.

Comparing the stimulatory indices of βTC6F7 cells in 18 and 66 nm pore-sized capsules *in vitro*, we find that the indices are approximately 4 and 9, respectively (Table 13.3.2). This suggests that the larger pores of the 66 nm capsules are more effective in facilitating nutrient diffusion to the encapsulated cells, leading to greater cell viability. This behavior is echoed in the case of RIN cells *in vitro*, where indices are approximately 2 and 6 for 18 and 66 nm biocapsules, respectively.

Turning to the explanted biocapsules, we see stimulatory indices of approximately 4 and 2 for βTC6F7 cells in 18 and 66 nm pore-sized capsules, respectively (Table 13.3.2). Similarly, RIN cells in explanted 18 and 66 nm pore-sized capsules both had indices of approximately 2. We observe that there is a

sharp reduction in the stimulatory indices of both cells lines in 66 nm biocapsules, whereas the stimulatory indices of explanted 18 nm capsules remains essentially the same as that which was observed for *in vitro* 18 nm biocapsules (Table 13.3.2). This seems to indicate the immunoisolatory effect of 18 nm pore-sized biocapsules and the lack of effective immunoisolation by the 66 nm biocapsules.

Table 13.3.3. Percent decrease in encapsulated cell insulin secretion (explanted/incubated) in microfabricated biocapsules

	% Decrease in Insulin Secretion between incubated and explanted biocapsules	
	66 nm capsules	18 nm capsules
RIN	66	22
βTC6F7	88	14

The decrease in levels of insulin secretion in the explanted biocapsules, compared to the incubated biocapsules is associated, in part, with a certain amount of shock induced in the implanted cells. The fact that 66 nm pore-sized capsules had a greater reduction in levels of insulin secretion than 18 nm capsules points to a scenario of negative feedback and shock, but *not* pore clogging, since one would expect bigger pores to clog less than smaller ones. This is also supported by the observation that both cell lines in implanted biocapsules had a decrease in insulin secretion compared to *in vitro*, despite the differences in immunogenicity. Since this was a short term study, the implanted cells may not have had a sufficient amount of time to adjust to their new environment and regain functional normalcy. These cells were then

explanted and subjected to another change in ambient conditions, resulting in yet further stress. Such shifts in environment could easily account for lower insulin secretory capabilities.

Since the experimental animals were normoglycemic, already established mechanisms of insulin feedback control may have altered or negatively inhibited insulinoma homeostasis. The implanted cells were augmenting and thus competing with fully-functioning pancreatic cells in terms of glucose-stimulated insulin secretion.

If we examine the data further, we find some interesting trends. For example, we can use the value of Be/Bi to predict Se/Si for both cell lines, where B = basal insulin secretion, S= stimulatory insulin secretion, e = explanted, and i = incubated. Looking at RIN cells in 18 nm capsules,

$$\left(\frac{B_e}{B_i}\right)\times 100 = 64.5\% \qquad (1)$$

$$\left(\frac{S_e}{S_i}\right)\times 100 = 47.7\% \qquad (2)$$

and $$\left[1-\frac{\left(S_e/S_i\right)}{\left(B_e/B_i\right)}\right]\times 100 \approx 25\% \qquad (3)$$

where 25% is a value representative of the % pore clogging.

Equation (3) can then be applied to βTC6F7 cells to predict the value of Se/Si. If Be/Bi = 3.7% and 1-(Se/Si)/(Be/Bi) ≈ 25% (from above), then Se/Si ≈ 2.8. This corresponds to the experimental value that was obtained. For 66 nm capsules,

the value for equation (3) is approximately 80%, and this corresponds to Se/Si values of 4.3% and 1.8% for RIN and BTC6F7 cells, respectively.

Additionally, perhaps the location and method of implantation could be improved. Biocapsules that did not migrate into lower body spaces seemed to perform better than those that did. If capsules themselves are placed in a larger sack or mesh to prevent movement or are anchored to tissue by suturing, this may improve performance and deter biocapsule adhesion to one another. Moreover, the rate of fibrosis seems to depend on the localization of the biocapsules. Limiting or preventing biocapsule displacement and migration will deter fibrotic capsule formation.

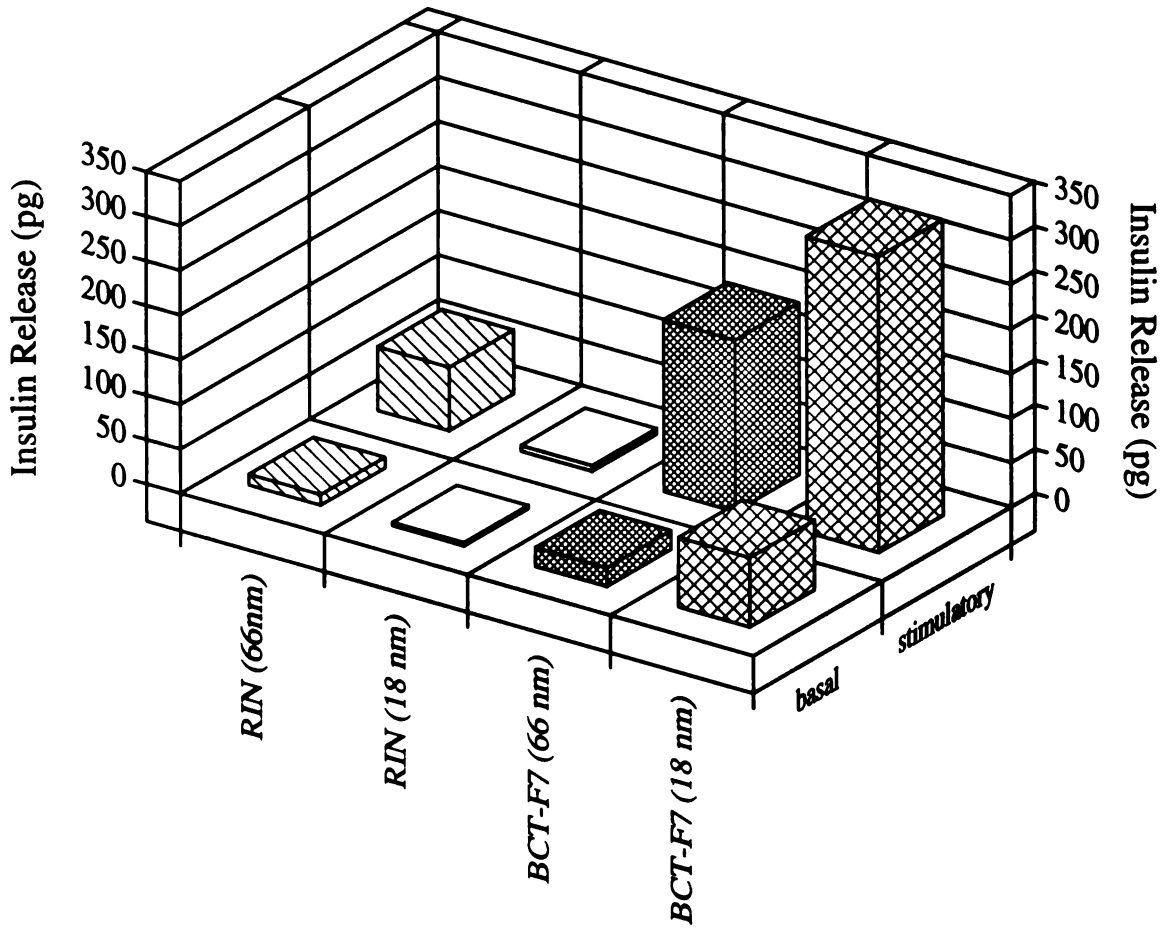


Figure 13.3.1 Insulin secretory response of encapsulated insulinoma cell in vitro

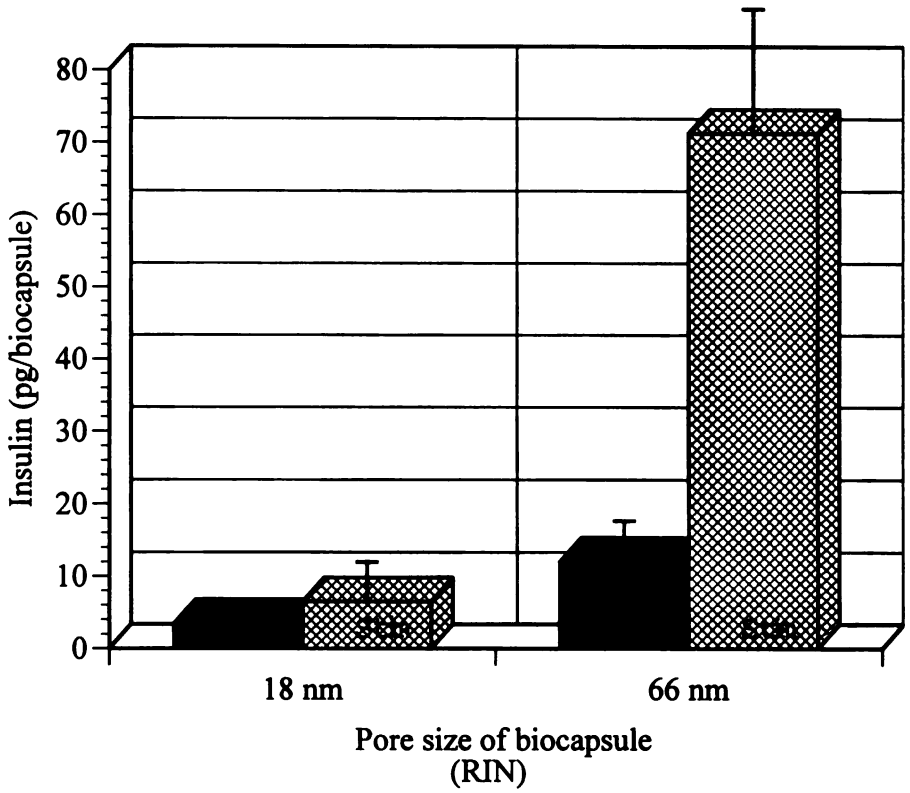


Figure 13.3.2 Basal and Stimulatory Insulin secretion from 18 and 66 nm pore sized biocapsules containing RIN cells incubated *in vitro* for one week

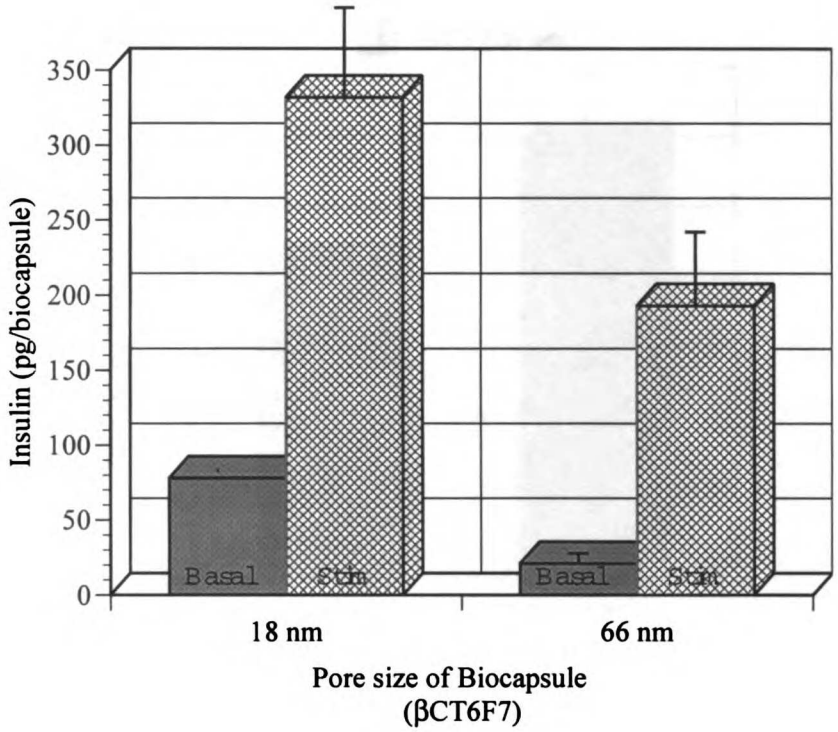


Figure 13.3.3 Basal and Stimulatory Insulin secretion from 18 and 66 nm pore sized biocapsules containing bTC6F7 cells incubated *in vitro* for one week.

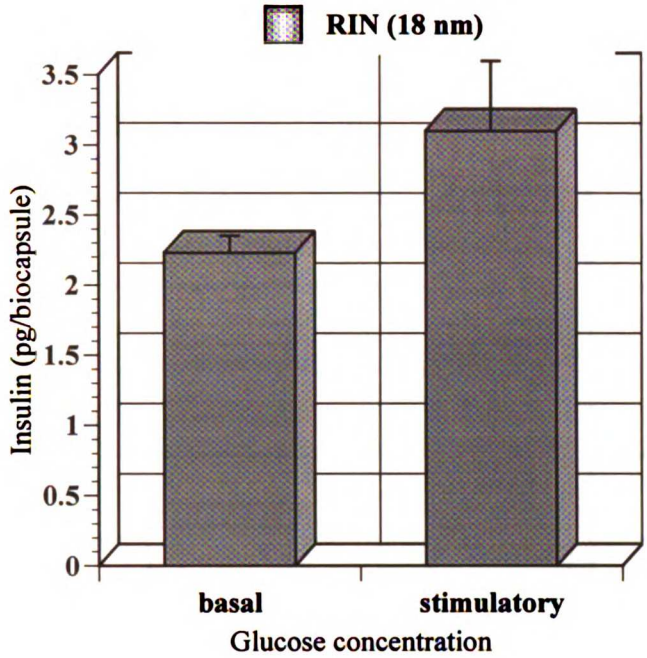


Figure 13.3.4 After 1 week of intraperitoneal implantation, glucose stimulated insulin secretion from the retrieved biocapsules with RIN cells was examined.

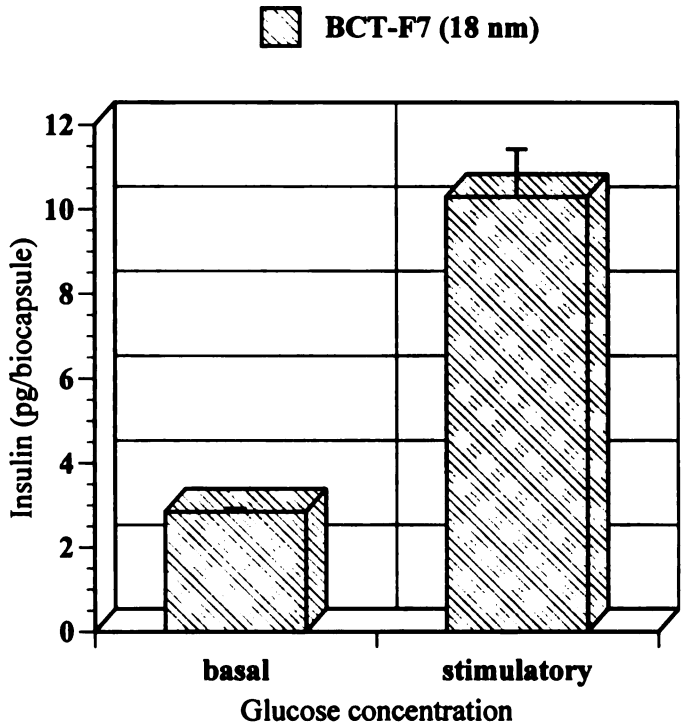


Figure 13.3.5 After 1 week of intraperitoneal implantation, glucose stimulated insulin secretion from retrieved biocapsules with βTC6F7 cells was examined.

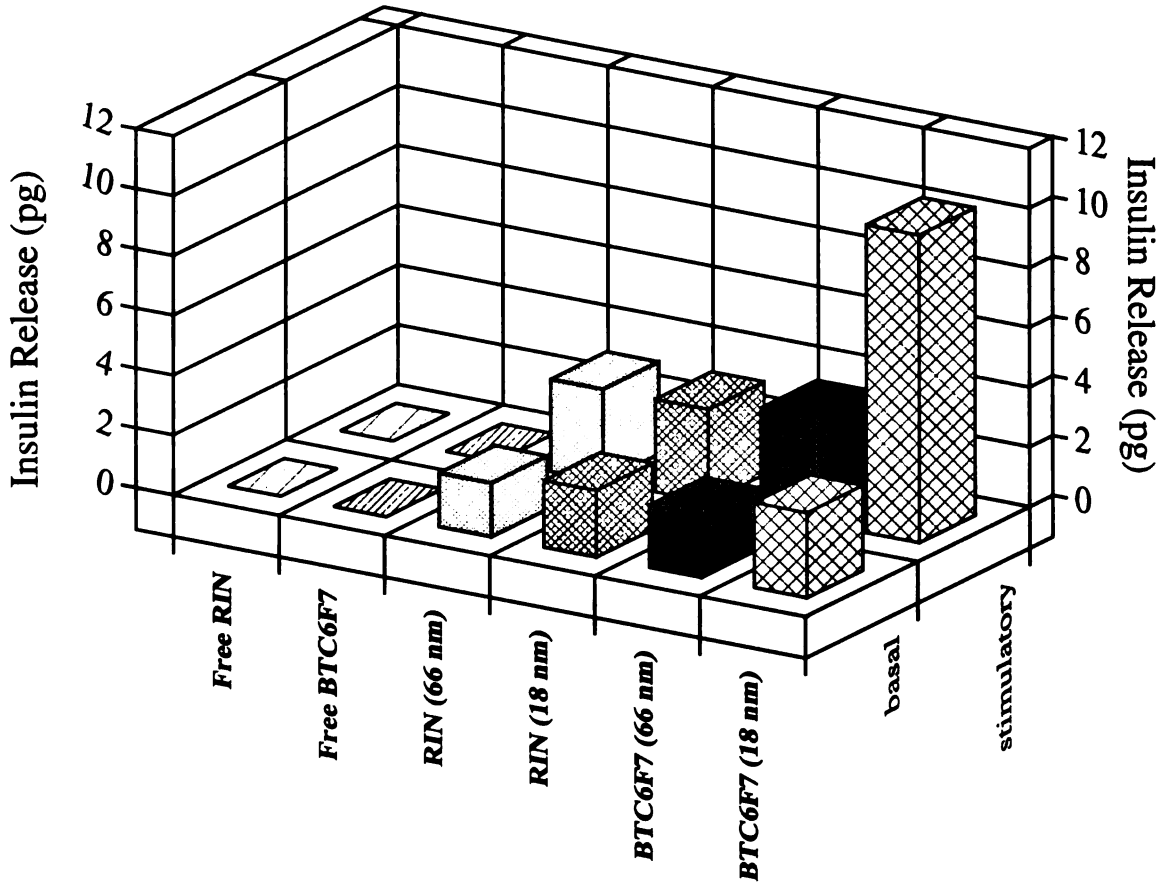


Figure 13.3.6 Insulin secretory response of encapsulated insulinoma cell upon explantation from BALB-C mice compared to freely injected insulinoma cells which were completely destroyed by the host's immune system.

13.4 Conclusions

A new biocapsule, made by microfabrication technology, has been developed for xenogeneic cell encapsulation. The results presented in this study demonstrate that after 1 week of intraperitoneal implantation retrieved microfabricated biocapsules maintained clean surfaces free from degradation or corrosion, and displayed no pore blockage. Stabilized biocapsules displayed no fibrotic encapsulation after one week implantation.

Both RIN and βTC6F7 insulinoma cell lines remained viable in the implanted biocapsules without the use of immunosuppressants and secreted insulin in response to both basal and stimulatory conditions. Differences in *in vitro* and *in vivo* levels of insulin secretion have been attributed to acute implant shock and a negative feedback mechanism due to the normoglycemic state of the mice. However, results indicate that 18 nm pore-sized biocapsules provide better immunoprotection of both cell lines. There was less than 20% decrease in stimulatory indices for the 18 nm biocapsules, compared to approximately 80% decrease 66 nm biocapsules. This decrease is attributed to insufficient immunoprotection rather than pore blockage, since 66 nm capsules would be expected to have less blockage and hence, greater insulin secretion. Nevertheless, all microfabricated biocapsules did provide some degree of immunoprotection, since unencapsulated insulinoma cells were rapidly and completely destroyed by the host's immune system.

Insulin secretion in response to glucose stimuli is an essential function of implantable biocapsules and maintenance of normoglycemia in general. The

microfabricated biocapsules that have been developed can serve to protect xenograft insulinoma cell lines from rejection and provide a stable microenvironment in which the cells can maintain insulin secretory responsiveness to glucose. Further studies will be needed to investigate whether microfabricated biocapsules encapsulating xenogeneic cells can reverse hyperglycemia in diabetic rats and larger animals.

13.5 Future Work

Although this project has yielded significant results regarding the promise of microfabrication technology for cellular therapeutics, many further studies can be performed to optimize and improve the performance of the biocapsule for the immunoisolation of islets, as well as other cell lines. This includes studies on pore geometry optimization, dose-response evaluation, and long term small and large animal studies.

The examination of microfabricated constructs with cell and tissue systems can be useful to the overall development of biohybrid organs and cell encapsulation devices. Being able to create model microfabricated constructs with parallel dimensions to physiological systems can aid in studying complex tissue interactions in terms of cell communication and response to matrix geometry and external fluid and chemical stimuli. Moreover, microfabricated drug and cell encapsulation systems can be further optimized through use of surface modification protocols for biocompatibility, substrate targeting, and

stimulation of angiogenesis using covalently bound growth factors and surface microarchitecture on silicon substrates.

The investigation of microfabricated biomaterial interfacial properties is critical to future advances in the fields of biotechnology and tissue engineering. By characterizing biocompatibility and tissue/cell suface modulation, and by creating precisely controlled three dimensional microenvironments to protect, stimulate, and enhance cell behavior, one can bring together synthetic and biological components in order to better understand their relationships and interactions. Most importantly, the microfabrication of three-dimensional microenvironments for cells using a variety of inorganic materials and microstructures opens up many new and exciting possibilities in terms of therapeutic treatment modalities using cell transplantation and drug delivery.

14. References

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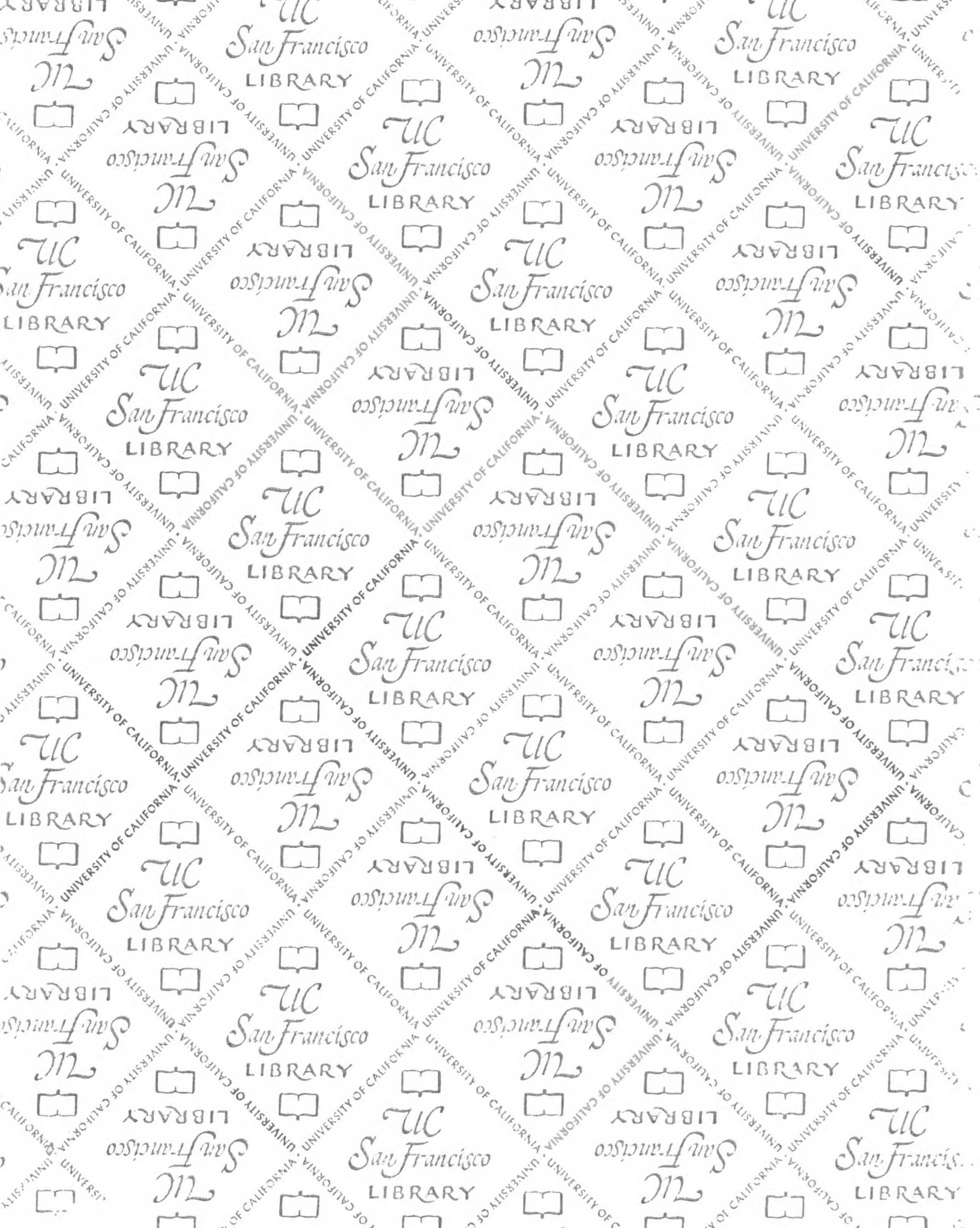
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